Intracellular signalling pathways activated by FcγRI

A thesis submitted to the University of Glasgow

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by

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ZAPATOS DE MI CONCIENCIA

Lo que digo en este canto
es la historia de un encuentro
que tuve con un anciano
y su antigua poesia
las dos rosas de sus manos.

Me dijo llegar temprano
es ver las cosas de frente
y ademas te queda tiempo
para mirarlas dos veces.

Al ver al viejo descalzo
quise darle mis zapatos
y me dijo no hace falta
lo que importa es tu conciencia
no es importante el ropaje
sino distinguir a fondo
los que van comiendo dioses
y defecando demonios.

Si tu vas por un camino
te gritan de lado y lado
mas no detengas tu marcha
tan solo para escucharlos
siempre encontraras la altura
en que todos puedan verte
y en la que puedas inclinarte
sin peligro de caerte,... zapatos.

El viejo tenia la piel
marcada por latigazos
pues la miseria de ayer
seguia saliendole al paso
y conto que en su pasado
no quiso ser dios ni heroe
en los dioses nadie cree
y a los heroes se condenan
a convertirse en estatuas
que ante las aves dan pena
de verdad que las estatuas
bajo las aves dan pena,... zapatos.

Zapatos de mi conciencia
mal que bien me van llevando.
Zapatos de mi conciencia
mal que bien me van llevando.

Ali Primera.

ABSTRACT

Intracellular signalling pathways activated by FcγRI

The high affinity receptor for immunoglobulin G, FcγRI, , plays a central role in host immune defence by linking the cellular and humoral arms of the immune system. However, the signalling pathways initiated by this receptor are poorly understood. In this thesis, the intracellular signalling pathways activated by immune complexes were studied using U937 cells as a model system to investigate the effect of differentiation on these pathways. Previous work has shown that, the nature and duration of the calcium transients initiated by immune complexes in these cells changes, as the cells differentiate into cells of a more macrophage phenotype. This thesis describes the signalling pathways underlying this change .

Marked differentiation dependent differences in the signalling pathways activated by immune complexes were found. Thus, in cells differentiated to a more macrophage phenotype with dbcAMP, Fc receptor aggregation resulted in the activation of PLC. In cells primed with IFN-γ, no activation of PLC could be detected. In contrast, immune complexes in these cells resulted in the activation of PLD.

The switch in the activation of intracellular signalling pathways was found to result from a switch in the use of accessory molecules by FcγRI. Thus, in IFN-γ primed cells, FcγRI signals through the recruitment of the γ chain whereas in dbcAMP differentiated cells FcγRI appears to recruit FcγRIIa.

A novel signalling pathway responsible for calcium transients and trafficking of immune complexes to lysosomes was identified. This pathway involved the sequential activation of PLD and sphingosine kinase with the generation of sphingosine-1-phosphate.

In these cytokine primed cells, immune complexes also resulted in the activation of PI3-kinase. However, an unusual pattern of PIP_3 production was observed. Both forms of Class I PI3-kinase were activated. Thus, the initial peak in PIP_3 resulted from the activation of the tyrosine kinase (p85) dependent form of PI3-kinase, whereas the latter, prolonged elevation of PIP_3, resulted from the activation of the βγ dependent form of PI3-kinase.

The switch in signalling pathway appears to dictate the isoform of PKC activated by FcγRI. Thus, in cytokine primed cells where PLD is activated and the resultant calcium transients are brief, only calcium independent isoforms were activated. In dbcAMP differentiated cells, where PLC activation results in prolonged calcium signals, only the calcium dependent PKC isoforms were activated.

PREFACE

The study described in this thesis was carried out between September 1995 and July 1998.

PUBLICATIONS RESULTING FROM THE WORK PRESENTED IN THIS THESIS

Chapter 2.

Alirio J. Melendez, R. Andres Floto, Angus J. Cameron, David J. Gillooly, Margaret M. Harnett, & Janet M. Allen.
A molecular switch changes the signalling pathway used by the FcγRI antibody receptor to mobilise calcium.
Current Biology. 1998, **8:** 210–221.

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Differentiation dependent switch in signalling pathways initiated by FcγRI.
Biochemical Society Transactions. 1997, **25**: 254S.

Alirio J. Melendez, Margaret M. Harnett, & Janet M. Allen.
FcγRI activates different phospholipid signalling pathways depending on cellular differentiation.
The Biochemical Society. Pre doctoral meeting. 1996, **Sep.**: A1.

Chapter 3

Alirio J. Melendez, R. Andres Floto, David J. Gillooly, Margaret M. Harnett, & Janet M. Allen.
FcγRI-coupling to phospholipase D initiates sphingosine kinase mediated calcium mobilisation and vesicular trafficking.
J. Biol. Chem. **273:** 9393–9400.

David J. Gillooly, **Alirio J. Melendez**, Margaret M. Harnett, & Janet M. Allen.
Vesicular trafficking of immune complexes and activation of Phospholipase D by FcγRI requires PI3–Kinase.
Submitted to *J. Exp. Immunol.* Currently under review.

Chapter 4

Alirio J. Melendez, Margaret M. Harnett, & Janet M. Allen.
Differentiation dependent switch in PKC isoenzyme activation by FcγRI the human high affinity receptor for immunoglobulin G.
Submitted to *Immunology*. Accepted for publication 15-Oct-98.

Chapter 5

Alirio J. Melendez, David J. Gillooly, Margaret M. Harnett, & Janet M. Allen.
Aggregation of the human high affinity immunoglobulin G receptor (FcγRI) activates both tyrosine kinase and G-protein coupled phosphoinositide 3-kinase isoforms.
Proc. Natl. Acad. Sci. USA. 1998, **95:** 2169–2174.

Alirio J. Melendez, David J. Gillooly, Margaret M. Harnett, & Janet M. Allen.
Distinct activation of two PI3-kinase isoforms by Fc receptors.
Scottish Signal Transduction Group. 1998, **Feb**.: 11.

Alirio J. Melendez, David J. Gillooly, Derek Boyle, Margaret M. Harnett, & Janet M. Allen.
The human high affinity receptor for immunoglobulin G is functionally coupled to activation of phosphoinositide 3-kinase
Physiological Society. Abstract to be published in *J. Physiol.*

DECLARATION

I hereby declare that this thesis embodies the results of my own special work*, that it has been composed by myself and that it does not include work forming part of a thesis presented for a degree in this or another University.

*This thesis consists of work published or to be published in the near future. The following collaborators have contributed to this work.

Chapter 2:
Dr. R. Andres Floto (University of Cambridge) – carried out the measurement of intracellular calcium release.
Mr. Angus J. Cameron (University of Glasgow) – did the Northern analysis.

Chapter 3:
Dr. R. Andres Floto (University of Cambridge) – carried out the measurement of intracellular calcium release.
Dr. David J. Gillooly (University of Glasgow) – carried out the trafficking experiments.

Date: 26 August 1998 Signature:

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DEDICATION

To my parents, Lilia and J.Exequiel,
to my parents-in-law, Liliane and Keith,
and especially to my wife Anneke.

Contents

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ABBREVIATIONS

Ab	Antibody
mAb	Monoclonal antibody
IgG	Immunoglobulin G
Fc	Constant region of immunoglobulins
F(ab)	Variable region of immunoglobulins
Fcγ receptors	Receptors for the constant region of IgG
FcγRI	High affinity receptor for IgG
FcγRIIa	Low affinity receptor for IgG
FcεRI	High affinity receptor for IgE
ITAM	Immunoreceptor tyrosine activation motif
ADCC	Antibody directed cellular cytotoxicity
dbcAMP	Dibutyryl cyclic AMP
IFN-γ	Interferon-γ
PtdChoPLD	Phosphatidyl choline phospholipase D
PtdInsP2PLC	Phosphatidyl inositol phospholipase C
InsP	Inositol phosphate
$InsP_2$	Inositl-bis-phosphate
$InsP_3$	Inositol-1,4,5-tris-phosphate
DAG	Diacylglycerol
DHS	D-L-*threo* dihydro-sphingosine
PMA	Phorbol 12,13 myristate acetate
PtdOH	Phosphatidic acid
PtdBut	Phosphatidylbutanol
PLC	Phospholipase C
PtdIns-PLC	Phosphatidylinositol-specific phospholipase C
PLD	Phospholipase D
PtdCho-PLD	Phosphatidylcholine-specific phospholipase D
PKC	Protein kinase C
PI3-Kinase	Phosphatidylinositol 3 kinase
PI	Phosphatidylinositol
PIP	Phosphatidylinositol-mono-phosphate
PIP_2	Phosphatidylinositol-bis-phosphate
PIP_3	Phosphatidylinositol-tris-phosphate
SP	Sphingosine
SPP	Sphingosine-1-phosphate
PBS	Phosphate buffered saline
HBS	Hepes buffered saline
TCA	Trichloroacetic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 Fc RECEPTORS; a case for study.

Perhaps the most important function of the immune system in host defence is the coordination and interplay between humoral and cell-mediated immune responses. Some of the proteins that are essential for this feedback of responses are the receptors for the Fc domain of immunoglobulins (FcRs). These proteins were initially discovered over 30 years ago and were known to be widely distributed on cells of the immune system. However, it is only in the last few years that the structure of these proteins and the genes that encode them have been elucidated, facilitating their study, and giving birth to a great amount of information.

1.1.1 Classes of Fc receptors

There are five isotypes of immunoglobulins (Igs) in mammals: IgA, IgD, IgE, IgG and IgM. There are particular Fc receptors which are usually specific for only one or two of the Ig isotypes. One class of Fc receptors transports immunoglobulins across epithelial tissues to their main site of action. This class includes the neonatal Fc receptor (FcRn) (Mostov & Simister 1985), which transports immunoglobulin G (IgG), and the polymeric immunoglobulin receptor (pIgR) which recognises dimeric IgA and pentameric IgM (Brandtzaeg 1981, reviewed in Mostov 1994). Another class of receptors are the lectin-like molecules that bind to IgE (Conrad 1990). However, the largest and best characterised group are the receptors that belong to the immunoglobulin gene superfamily: these receptors are type I glycoproteins present on the surface of effector cells. This group includes

receptors specific for IgG (FcγR), for IgE (FcεR), and IgA (FcαR) (Maliszewki 1990). Furthermore Fc receptors specific for IgM (FcμR) (Ohno *et al* 1990) and IgD (FcδR) (Sjoberg 1980) have been found.

The focus of this thesis is the study of the type I glycoprotein receptors present on the surface of effector cells that bind the Fc region of IgG (FcγRs).

1.1.2 Different IgG Fc receptors (FcγRs).

Three clones of FcγRs were defined by affinity, tissue distribution and monoclonal antibody recognition. This revealed the existence of three distinct, but closely related, FcγR classes: FcγRI(CD64), FcγRII (CD32) and FcγRIII (CD16). Molecular cloning of the genes encoding these proteins have revealed that each subclass contains a variety of isoforms. Thus in man there are three genes for FcγRI, three genes for FcγRII and two genes for FcγRIII (reviewed in Hulett & Hogarth 1994, Ravetch & Kinet 1991). All these receptors are membrane glycoproteins composed of a ligand-binding α subunit, comprising highly conserved extracellular Ig-like domains, with identity ranging from 70%-98%; a polypeptide or lipid anchor in the membrane; and a cytosolic domain that is not very conserved between receptors. (Figure 1) (reviewed in Ravetch & Kinet 1991, Burton and Woof 1992, Hulett & Hogarth 1994).

1.1.3 FcγR; the α subunits

The α subunits of several FcγRs are found in multiple forms (Ravetch *et al* 1986, Stuart *et al* 1989, Qiu *et al* 1990, Ersnt et al 1992; reviewed in Ravetch & Kinet 1991, Friedmann *et al* 1992, Hulett & Hogarth 1994). In humans, three FcγRI, three FcγRII, and two FcγRIII genes have been identified (reviewed in Ravetch 1994, Hulett & Hogarth 1994).

1.1.4 Structure of the FcγRs ligand-binding subunit

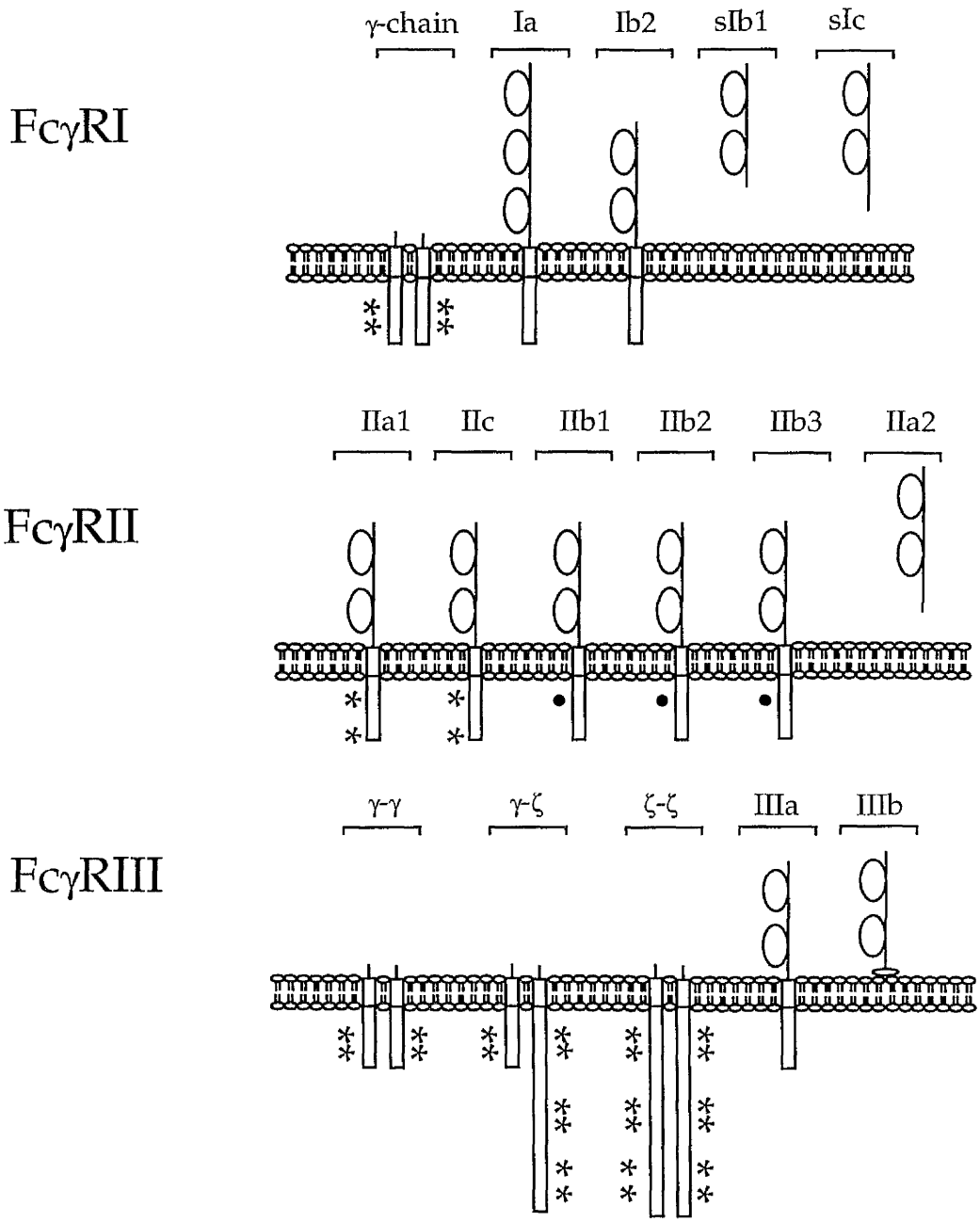
The Ig-binding subunit of FcγRs are generally thought of as being monomeric based on analogy to similar Ig superfamily structures including CD4, CD2, and VCAM-1 (Ryu *et al* 1990, Wang *et al* 1990, Jones *et al* 1992 & 1995). In agreement with this, it has been shown that FcγRI binds only a single IgG molecule (O`Grady *et al* 1986), although this does not conclusively demonstrate that it is a monomer.

1.1.5 Proteins required for FcγR assembly and signalling (the γ and ζ chains)

The ligand-binding subunits of some FcγRs are associated in the membrane with other proteins which are required for receptor signalling. The α chain of FcγRI associates with an integral membrane protein called the γ chain (Ra *et al* 1989, Ernst *et al* 1993, Scholl & Geha 1993). The γ chain is homologous to the ζ chain, a protein originally identified as essential for the assembly and signalling of the T-cell receptor-CD3 complex (Weissman *et al* 1989). Both γ and ζ chains associate with FcγRIII, and expression of the γ chain is essential to support surface expression of FcγRIII. (Figure 1) (Hibbs *et al* 1989, Lanier *et al* 1989, Orloff *et al* 1990).

Figure 1

Fcγ receptors and the signalling molecules with which they interact



FcγR Ia; Ib2; IIa1; IIb1; IIb2; IIb3; IIc; and IIIa are transmembrane proteins, whereas FcγR sIb1; sIc; IIa2 are soluble proteins and FcγRIIIb is a GPI-anchored protein.

FcγRIa and IIIa receptors do not contain an ITAM and therefore associates with the signalling molecules γ and/or ζ chains in order to signal.

FcγRIIa1 contains an ITAM of its own, and the FcγRIIb proteins contain an inhibitory motif (ITIM).

* denotes Tyr residues within the ITAMs

• denotes Tyr residues within the ITIMs.

1.1.6 ITAMs

The cytoplasmic domains of γ and ζ chains share a common tyrosine-containing sequence motif called the immuno-receptor tyrosine activatory motif (ITAM) (Cambier 1995), previously variously known as ARAM (Reth 1989, Weiss & Littman 1994), TAM (Samelson & Klausne 1992), or the ARH1 motif (Cambier 1992).

The motif consists of six conserved amino acid residues spaced precisely over an ~26-amino acid sequence ($D/EX_7D/EX_2YX_2LX_7YX_2L$). This is the case with the γ chain, and the ζ chain. However, there is one small variation in this motif and it is the ITAM for FcγRIIa, which has 12 instead of 7 amino acid residues between the $YX_2L - YX_2L$ in the motif ($D/EX_7D/EX_2YX_2LX_{12}YX_2L$).

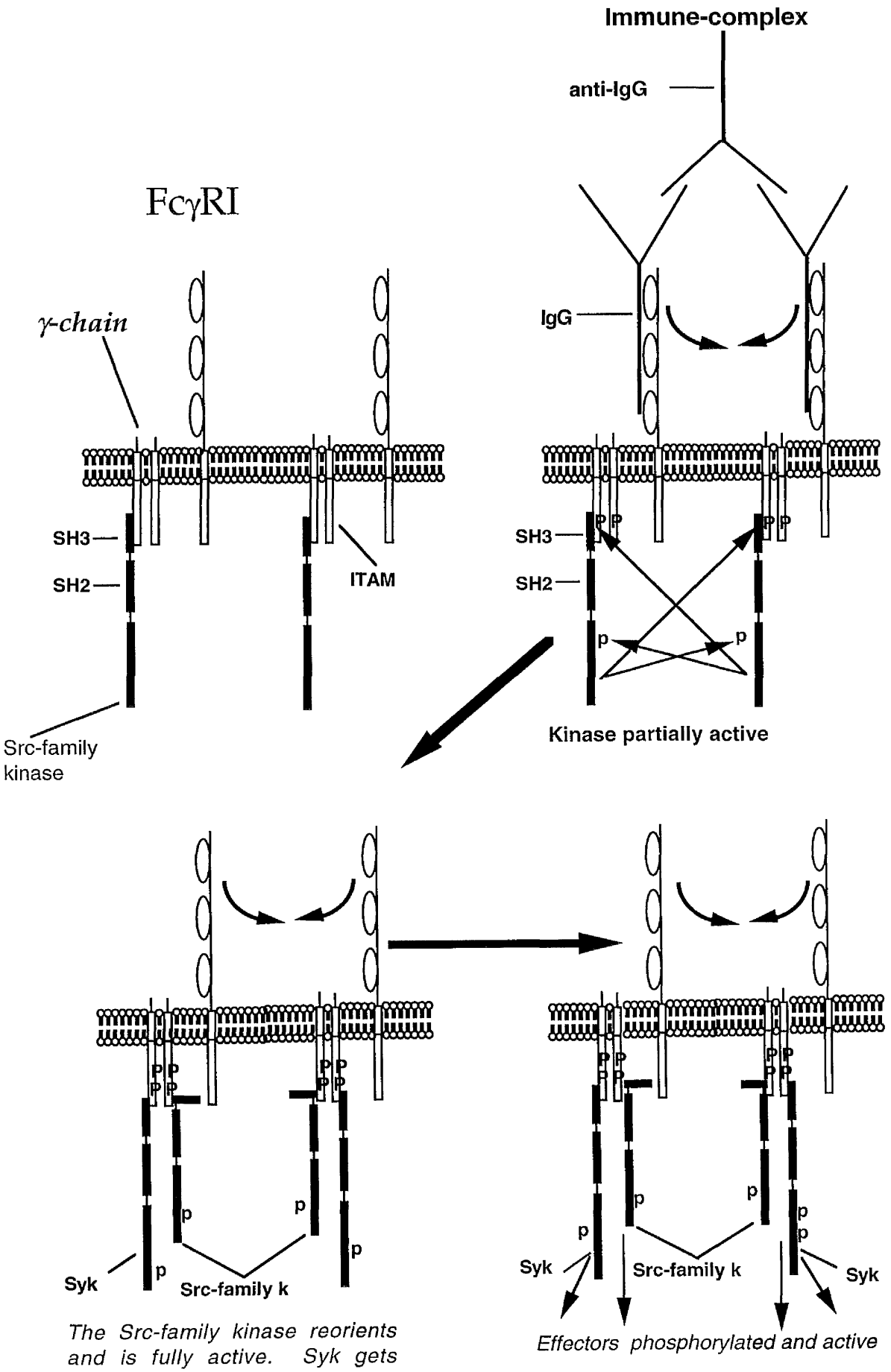
1.1.7 Signals generated by FcγRs: a receptor activation model

Cross-linking of FcγRs on cells such as macrophages, neutrophils, and NK cells results in the transduction of signals leading to: the activation of tyrosine kinases; elevation of intracellular calcium; release of inflammatory mediators such as leukotrienes, prostaglandins, and hydrolases; and the transcription of genes encoding cytokines (van de Winkel & Capel 1993, Edberg *et al* 1995, Deo *et al* 1997). These cellular responses are similar to those observed when the antigen receptors of T cells or B cells are cross-linked. This similarity is thought to be due to the homology of the signalling motifs in the cytoplasmic domain of all these receptors. These motifs are collectively known as the Immunoreceptor Tyrosine-Based Activation Motif (ITAM). The general scheme that has been deduced for TCR and BCR signalling turns out to be the same for FcR signalling as well (Cambier 1995, Johnson *et al* 1995, Agarwal *et al* 1993). In the model proposed by Cambier (1995), the ITAM associates with an inactive tyrosine kinase of the src family: cross-linking of the extracellular domain of the receptor results in the activation of the kinase and

phosphorylation of two critical tyrosines found in the ITAM. The phosphorylated ITAM is then able to interact with another class of tyrosine kinases, such as syk, (zap–70 in TCR), through SH2 interactions with the ITAM. The complex thus formed goes on to phosphorylate intracellular substrates, like phospholipase C-γ1 and Phosphatiydylinositol 3-kinase, and initiate effector functions and the subsequent biological responses triggered by the cells. (Figure 2).

Figure 2

Model for Fc-receptor activation



The Src-family kinase reorients and is fully active. Syk gets recriuted to the complex

Effectors phosphorylated and active

1.1.8 Biological responses triggered by FcγRs

Effector responses mediated by FcγRs include phagocytosis, endocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and the release of inflammatory mediators, (Reviwed in Unkeless *et al* 1988, van de Winkel & Anderson 1991).

1.1.8.1 Phagocytosis

Phagocytosis is the ability to internalise large particules 1 μm or greater in diameter (Silverstein *et al* 1977). Uptake is triggered by binding of opsonised particles to cell surface receptors capable of transducing a phagocytic stimulus to the cytoplasm. This stimulus results in the localised polymerisation of actin at the site of particle attachment and subsequent pseudopod extension that engulfs the bound particle into a cytoplasmic phagosome (Greenberg *et al* 1990, 1991, Allen 1996). In macrophages, the forming pseudopods are directed by sequential ligand-receptor interactions, which yield a vacuole custom–fit to the internalised particle (Silvertein *et al* 1977). The phagosomes are able to fuse with endosomes and/or lysosomes exposing their contents to lower pH and hydrolytic enzymes (Rabinowitz *et al* 1992, Desjardins *et al* 1994, Allen 1996). The degraded product can then be processed and presented on the cell surface as degraded product-derived peptides, via class II MHC molecules, to antigen-specific T lymphocytes (Harding & Geuze 1992, Pfeifer *et al* 1993).

1.1.8.2 Endocytosis

Endocytosis is the process of internalising small particles (<0.2 μm in diameter), such as, immune complexes (antigen–antibody). Most receptor-ligand complexes accumulate at clathrin-coated pits of the plasma membrane, which bud off to yield clathrin-coated vesicles. The vesicles rapidly lose their coats, which facilitates fusion with early endosomes (EEs); a dynamic array of

tubules and vesicles distributed throughout the peripheral and perinuclear cytoplasm. Due to a slightly acidic pH (pH~6.0-6.8) maintained by an ATP-driven proton pump (Mellman *et al* 1986, Forgac 1992), EEs host the dissociation of many ligand-receptor complexes. Free receptors selectively accumulate in the early endosome's tubular extensions, which bud off to yield recycling vesicles (RVs) that transport receptors directly or indirectly back to the plasma membrane. Dissociated ligands collect in the vesicular portions of the EEs because of their high internal volume relative to the endosome's tubular extensions. The vesicular structures pinch of or are left behind following the budding of RVs traverse to the perinuclear cytoplasm on microtubule tracks, and fuse with late endosomes (LEs) and lysosomes. Here, ligands are degraded by an even lower pH (pH~5) and the high concentration of lysosomal enzymes. Endocytosis is thought of as having two different functionally and physically distinct compartaments (Kornfeld & Mellman 1989, Mellman 1996). Early endosomes are responsible for the dissociation and sorting of receptors and ligands in an environment that minimizes the risk of damaging receptors intended for reutilisation. Late endosomes and lysosomes are responsible for accumulating and digesting exogenous and endogenous macromolecules (Mellman 1996, Robinson *et al* 1996).

1.1.8.3 ADCC

Antibody-dependent cell-mediated cytotoxicity (ADCC). FcγRs present on cells such as natural killer cells mediate interactions with antibody-coated target cells, resulting in the destruction of target cells via NK cells in the process known as ADCC (van de Winkel & Anderson 1991)

1.1.9 FcγRI expression and genes

FcγRI is expressed on the surfaces of monocytes, macrophages and IFN–γ stimulated neutrophils (van de Winkel & Anderson 1991, Hullet & Hogarth 1994). Three human FcγRI genes have been identified, hFcγRIA, hFcγRIB, hFcγRIC (that generate four isoforms; see Figure 1), and mapped to the chromosome 1q21.1. hFcγRIA encodes a transmembrane receptor with three Ig-like domains; hFcγRIB and hFcγRIC encode soluble receptors with three Ig-like domains (Figure 1). Alternative splicing of one of the soluble receptor genes results in an mRNA for a transmembrane receptor with two Ig-like domains (Ernst *et al* 1992).

1.1.9.1 FcγRI; the only FcγR that binds monomeric IgG

This receptor binds monomeric IgG with high affinity; with K_A values ranging from $2 \times 10^9\ M^{-1}$ (for the binding of human IgG1 to human monocyte-like U937 cells expressing endogenous FcγRI; Shopes *et al* 1990) to $5 \times 10^9\ M^{-1}$ (for the binding of human IgG1 to COS cells transfected with human FcγRI; Allen & Seed 1989). The unique role of FcγRI compared with the low-affinity receptors FcγRII and FcγRIII may lie in the capability of FcγRI to trigger effector responses at low IgG concentrations, which are typical of early immune responses in vivo (Shen *et al* 1987). The α chain of FcγRI consists of three extracellular Ig-like domains, a transmembrane region, and a cytoplasmic domain (Allen & Seed 1989, Sears *et al* 1990, Ernst *et al* 1992). The α chain associates on cell surfaces with a γ homodimer protein (γ chain) (Scholl & Geha 1993, Ernst *et al* 1993). (Figure 1).

1.1.9.2 FcγRI interacts better with IgG because of an extra Ig-like domain

FcγRI has three extracellular Ig-like domains, unlike FcγRII and FcγRIII (lower affinity receptors) which possess two domains. Domains 1 and 2 of FcγRI share greater sequence similarity with the two extracellular domains of FcγRII

and FcγRIII than does domain 3, which suggests that the third domain is responsible for some of the interactions which make FcγRI a high affinity receptor for IgG and the only one that binds monomeric IgG at physiological concentrations (Allen & Seed 1989). It has been shown that removal of the third domain of murine FcγRI abolishes high-affinity binding to monomeric IgG, although domains 1 and 2 on their own retain a weak affinity for IgG. However, linking FcγRI third domain to domains 1 and 2 of FcγRII does not give high-affinity binding of monomeric IgG to this FcγRII chimera (Hulett *et al* 1991, Harrison & Allen 1988). This is telling us that there must be regions of FcγRI in addition to the third extracellular domain which are required for the high-affinity binding capacity of FcγRI. Use of receptor chimeras between FcγRI and FcγRII demonstrate that the second domain of FcγRI is essential for high binding binding (Harrison & Allen 1998)

1.1.9.3 Biological functions mediated by FcγRI

FcγRI mediates endocytosis and phagocytosis (Shen *et al* 1987, CL Anderson *et al* 1990, Davis *et al* 1995). Although FcγRI is capable of binding monomeric IgG with high affinity, the signals necessary for endocytosis and phagocytosis are generated only upon receptor cross-linking (Davis *et al* 1995). However, binding of FcγRI with monomeric IgG results in a transient internalisation of receptor-IgG complexes, in which the receptor-IgG complexes are rapidly recycled back to the cell surface (Harrison *et al* 1994). Cross-linking FcγRI at the cell surface promotes internalisation, the receptor-ligand complexes are then retained in intracellular endocytic compartments and trafficked to lysosomes for degradation (Mellman & Plutner 1984, Harrison *et al* 1994, Norman *et al* 1998). Endocytosis of cross-linked FcγRI-immune-complexes could lead to the enhanced presentation of peptide antigens on class II MHC

molecules, as described for FcγRIII-immune complex interactions (Amigorena *et al* 1992).

1.1.10 FcγRII expression and genes

FcγRII is widely distributed among cells of the immune system; it is found in monocytes, macrophages, neutrophils, basophils, B cells, Langerhans cells, platelets, and endotelial cells (placenta) (van de Winkel & Anderson 1991, Hulett & Hogarth 1994). Three FcγRII genes have been identified: hFcγRIIA, hFcγRIIB, and hFcγRIIC, located on chromosome 1q23-24 (Ravetch *et al* 1986, Brooks *et al* 1989, Stuart *et al* 1989, Qiu *et al* 1990), encoding multiple transcripts (Figure 1) that differ primarily in their cytoplasmic tails (Ravetch & Kinet 1991) . The α chains of human and murine FcγRII contain two extracellular Ig-like domains connected to a transmembrane and cytoplasmic region (Figure 1) (Ravetch *et al* 1986, Brooks *et al* 1989, Stuart *et al* 1989).

1.1.10.1 FcγRII binds IgG with low affinity

FcγRII binds monomeric IgG with low affinity (estimated $K_A < 1 \times 10^7 M^{-1}$ for hFcγRII binding to IgG; Hulett & Hogarth 1994). Under physiological conditions, the low affinity of FcγRII for monomeric IgG ensures that this receptor interacts only with IgG that has been aggregated by binding to multivalent antigens.

1.1.10.2 Biological functions mediated by FcγRII

The FcγRII proteins are an example of how distinct biological responses can be elicited by differences in the cytoplasmic domains of receptors that have common extracellular ligand-binding domains. Human FcγRIIA protein (FcγRIIa) contains an ITAM within their cytoplasmic domain which allows these cells to mediate conventional cellular activities (e.g. phagocytosis and

endocytosis) upon receptor aggregation (van der Herik-Oudijk *et al* 1995, Ravetch 1994, van de Winkel & Capel 1993). However FcγRIIB protein (FcγRIIb) functions *in vivo* as an inhibitory receptor for both B and mast cells (Takai *et al* 1996). This FcγRII isoform that has inhibitory effects does not contain an ITAM, instead it contains an inhibitory motif within its cytoplasmic domain (ITIM) (Muta *et al* 1994, Daeron 1995, Takai *et al* 1996). Both *in vitro* and *in vivo* studies have shown that FcγRIIb acts as a negative regulator of immune complex-triggered activation (Muta *et al* 1994, Daeron 1995, Takai *et al* 1996).

1.1.11 FcγRIII expression and genes

FcγRIII is expressed on macrophages, neutrophils, mast cells, and is the only Fc receptor found on natural killer cells (van de Winkel & Anderson 1991, Hulett & Hogarth 1994). Two human FcγRIII genes have been identified; FcγRIIIA and FcγRIIIB, mapped to chromosome 1q23-24. Both encode proteins that contain an extracellular portion of 180 amino acids with two Ig-like domains (Ravetch & Perussia 1989). The most significant difference between FcγRIIIA and FcγRIIIB genes is that the FcγRIIIA gene encodes FcγRIIIa, a protein with a polypeptide transmembrane region and a cytoplasmic domain of 25 amino acids, whereas the FcγRIIIB gene encodes FcγRIIIb, a protein that is anchored to the membrane by a glycophosphatidyl inositol linkage (Figure 1) (Ravetch & Perussia 1989).

1.1.11.1 Different cellular distribution of FcγRIII proteins

FcγRIIIa and FcγRIIIb have different cellular distributions; FcγRIIIa is expressed on macrophages, natural killer cells, and mast cells, whereas FcγRIIIb is expressed mainly on neutrophils (van de Winkel & Anderson

1991, Hulett & Hogarth 1994). FcγRIIIa α chain associates with the γ as well as ζ chains (Hibbs *et al* 1989, P. Anderson *et al* 1990).

The γ and ζ chains protect the FcγRIII α chain from degradation in the endoplasmic reticulum, and the absence of associated γ or ζ chains results in a reduction in cell surface expression of the transmembrane version of FcγRIII (Hibbs *et al* 1989, P. Anderson *et al* 1990).

1.1.11.2 Biological functions mediated by FcγRIII

FcγRIII mediates ADCC: the *in vivo* role of FcγRIII in mediated ADCC was examined by studying γ-chain-deficient mice. Natural killer cells from γ-chain-deficient mice cannot mediate ADCC because of the absence of cell surface FcγRIII (Takai *et al* 1994). In addition to its role in ADCC, FcγRIII functions in endocytosis and phagocytosis (van de Winkel & Anderson 1991, Amigorena *et al* 1992b, Daeron *et al* 1994, Nagarajan *et al* 1995).

1.2 SIGNALING BY PROTEIN TYROSINE PHOSPHORYLATION

1.2.1 Protein phosphorylation

Protein phosphorylation is a rapidly reversible reaction that represents a general mechanism for the regulation of intracellular events in response to environmental changes (Hunter *et al* 1985, Cantley 1991, Sonfyang & Cantley 1995). The genes encoding a large number of serine/threonine-specific, tyrosine-specific, or dual-specificity protein kinases have been cloned and compared (Hanks & Hunter 1988, Hunter 1995), and it is clear, on the basis of sequence conservation at the catalytic domain, that both protein serine/threonine kinases and tyrosine kinases evolved from a common

ancestor. They then diverged to recognise distinct sets of substrates in the cell, thereby regulating distinct cellular responses.

1.2.2 Protein-tyrosine kinases

Protein tyrosine kinases are involved in many signal tranasduction systems such as cell proliferation and differentiation (Hunter & Cooper 1985, Ullrich & Schlessinger 1990, Cooper 1990). These kinases can be divided into receptor-type tyrosine kinases (RTK), and non-receptor-type protein tyrosine kinases (NRPTK). The latter is the type of kinases involved in signalling by immune receptors.

1.2.3 Receptor tyrosine Kinases

RTK are cell surface proteins that are receptors for ligands, such as the receptors for many growth factors; a classical example is the insulin receptor (Kahn & White 1998). Insulin binding regulates cellular growth and metabolism through the activation of the integral tyrosine kinase in the β-subunit of the insulin receptor (Kahn & White 1998). Insulin-stimulated receptor autophosphorylation activates the kinase leading to tyrosyl phosphorylation of endogenous substrates (such as PLC-γ and PI3-kinase) which play a role in signal transduction cascades by generating second messengers (Kasuga *et al* 1983, Rosen *et al* 1993, White *et al* 1985, Wilden *et al* 1990, Backer *et al* 1992)

1.2.4 Non-receptor protein tyrosine kinases (NRPTKs)

NRPTK are intracellular protein tyrosine kinases that fall into two classes. src-family protein tyrosine kinases (PTKs), and syk and zap–70 PTKs (Taniguchi *et al* 1991, Burkhardt *et al* 1991).

1.2.4.1 src-like kinases

src-like kinases (src, lyn, fyn, yes, hck, blk, lck, abl, crk and nck) are widely distributed among haematopoetic cells with some degree of cell type specificity. These kinases possess one src-homology region 2 (SH2) domain, one SH3 domain and a catalytic domain (reviewed in Mustelin & Burn 1993). These kinases are anchored at the interface of the plasma membrane by myristoylation of the N-terminal glycine residue (Bolen *et al* 1992).

1.2.4.2 src family kinases in immune-receptor signalling

src family kinases act as signal transducers in association with cell surface receptors that lack an intracellular catalytic domain (Cooper 1990, Semba & Toyoshima 1990). This concept was originally established by the observation that the src-like kinase lck is physically and functionally associated with the T-cell surface antigens CD4 and CD8, receptors for major histocompatibility complex molecules (Veillette *et al* 1989, Barber *et al* 1989, Abraham *et al* 1991). Associations of the interleukin 2 receptor β chain and T-cell antigen receptor with the src-like kinases lck and fyn respectively, have also been reported (Hatakeyama 1991, Samelson 1990, Cooke 1991).

1.2.4.3 syk and zap-70 kinases

syk kinase is expressed in B and monocytic cells (Hutcherof *et al* 1991) and its primary structure is highly homologous to zap-70 which is expressed in T and NK cells (Chan *et al* 1991, 1992). Unlike the src-like kinases, syk and zap–70 bear two SH2 domains and a catalytic domain, but no myristoylation site (Tanguchi *et al* 1991, Chan *et al* 1992).

1.2.4.4 syk and zap–70 kinases in immune-receptor signalling

These kinases act in signal transduction by binding, presumably, to the ITAM motif of receptor subunits. Based on the analysis of association between zap-70 and the ζ chain through TCR stimulation (Chan *et al* 1991, 1992), it is hypothesised that zap-70 binds phosphorylated tyrosine residues of the ζ chain via SH2 domains once the ζ chain has been phosphorylated, which occurs through a src-like kinase, after receptor stimulation (Weiss 1993). However, in the case of syk activation, some discrepancy has been found: in B cells the syk kinase has been reported to associate with the BCR complex even in the absence of ligand (Hutchcroft *et al* 1992).

1.2.5 Protein dephosphorylation (role of phosphatases)

As mentioned in section 1.2.1, protein phosphorylation is a rapidly reversible reaction that represents a general mechanism for the regulation of intracellular events. The role of protein phosphorylation and of PTKs in generating signals has already been discussed. However, dephosphorylation is another very important regulatory factor in intracellular signalling. Membrane–bound phosphatases have been shown to play two distinct regulatory roles in PTK-dependent signalling by immune receptors. It has been proposed that PTKs, loosely associated with non-aggregated receptors, are prevented from signalling by the presence of membrane-bound phosphatases (Beaven & Metzger 1993). However, in some systems the opposite seems to happen (Mustelin et al 1992), creating some degree of controversy.

1.2.5.1 Role of protein phosphatases in immune-receptor signalling

CD45, a membrane–bound phosphatase, has been shown to inhibit FcγR signalling when co-aggregated (Rankin *et al* 1993). In contrast, CD45 has been shown to be essential for TCR activity (reviewed in Mustelin & Burn 1993).

In isolated T cell membranes and *in vivo*, both lck and fyn can be activated by CD45-mediated dephosphorylation (Mustelin *et al* 1989, 1992). In addition, fyn and lck activities were markedly reduced in both CD45-negative cells and in normal T cells treated with the tyrosine phosphatase inhibitor, phenylarsine oxide (Biffen *et al* 1994). It has been proposed that the catalytic activity of fyn and lck is activated by dephosphorylation (through CD45) and inhibited by phosphorylation (through the cytosolic PTK, p50*csk*) of a negative regulatory tyrosine residue (Mustelin & Burn 1993). Thus, phosphatases appear to either inhibit or enhance the activity of different PTKs and may, therefore, play a critical role in modulating the resultant PTK-dependent signalling pathways. However, the physiological role that CD45, or other phosphatases, may play in Fc-receptor signalling has yet to be elucidated.

1.3 LIPID SIGNALLING

In response to the activation of cell surface receptors, the levels of free lipids increase 1.4-2-fold above control levels (Nishizuka 1992 & 1995, Hannun 1994, Kolesnick 1994). These quantities greatly underestimate the increase in the signalling pools, which are generally considered to contain little or no second messenger in the absence of stimulus. Unfortunately, conventional assays cannot distinguish between the structural and signalling pools of these lipids and measure stimulated elevations over the entire cell.

Signal generation is followed by signal amplification, an observation that helps to explain how small transient signals generated at the cell surface can induce a wide range of biological responses. A consensus is emerging that recognises the interaction of lipid second messengers and protein kinase

cascades. In this regard, many lipid second messengers have the ability to cross bilayers and connect compartments that are normally separated under resting conditions.

The initial and most important step in the generation of lipid or lipid derived second messenger is the hydrolysis of phospholipids by a group of enzymes called phospholipases, and also by the phosphorylation of lipids by lipid kinases.

1.3.1 Phospholipases C

The phosphoinositide-specific phospholipases C (PLCs) are enzymes that specifically hydrolyse phosphatidylinositol 4-5-bisphosphate (PIP_2) to generate the second messengers diacylglycerol (DAG) and inositol-1-4-5-trisphosphate (IP_3) (Lee & Rhee 1995). (Figure 3).

1.3.1.1 Classes of PLCs

Three different groups of PLCs have been identified: β; γ and δ (Lee & Rhee 1995). All three PLC isoforms are single polypeptides containing two regions of high homology, designated X and Y, preceded by a plekstrin homology (PH) domain. In addition, PLC-γ has multiple src homology region 2 (SH2) domains and a single SH3 (Lee & Rhee 1995). (Figure 4).

1.3.1.2 Mechanisms for PLC activation

Two mechanisms have been described for PLC activation: PLC-β activation is mediated via heterotrimeric G proteins, in G-protein-coupled receptors. PLC-β activation occurs via GTP-bound α subunits of the Gq class or via βγ subunits. Gqα interacts with a region distal to the Y domain whereas the βγ subunits bind to an amino-terminal segment, perhaps via the PH domain (Lee & Rhee 1995). On the other hand, PLC-γ binds to receptor tyrosine

kinases or receptor-associated tyrosine kinases via SH2 domains and is activated by phosphorylation. (Figure 5).

1.3.1.3 Role of diacylglycerol (DAG) and inositol-1-4-5-trisphosphate (IP_3) in signal transduction

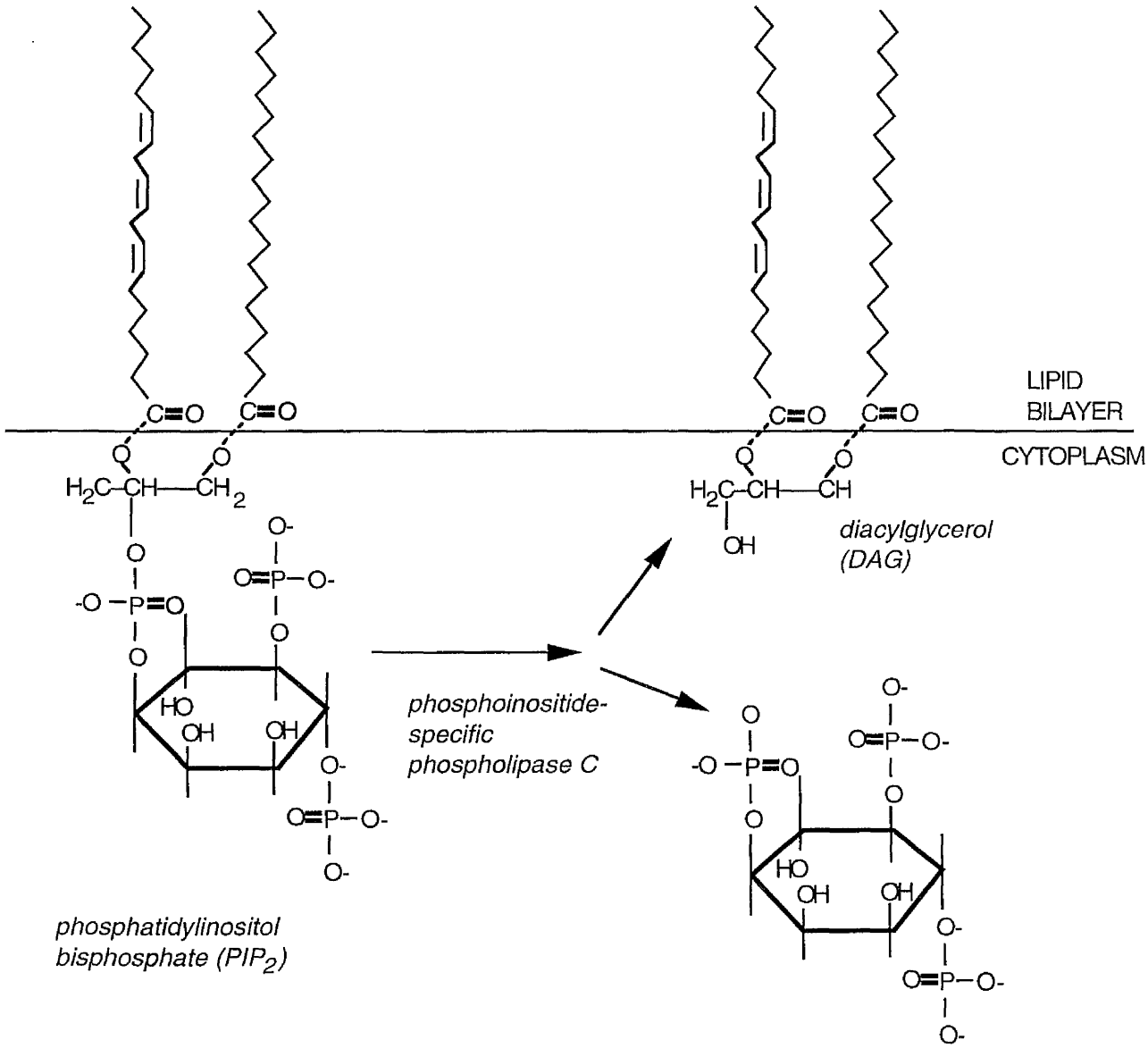
Diacylglycerol (DAG) acts as a second messenger by activating a number of the serine/threonine protein kinases (PKCs) (Nishizuka 1992 & 1995); whereas inositol-1-4-5-trisphosphate (IP_3) binds to an IP_3 receptor (IP_3R) to mobilise intracellular stored calcium and to promote an influx of external calcium, perhaps working in conjunction with IP_4 (Irvine 1990, 1991). (Figure 5).

1.3.1.4 Role of phosphatidylinositol transfer protein in PLC signalling

A major finding, in the lipid signalling pathways, is the recognition of the role of phosphatylinositol transfer protein (PITP) in signalling through either PLC-β or PLC-γ (Thomas *et al* 1993, Kauffmann-Zeh *et al* 1995). Although PITP transfers phosphatidylinositol (PI) and not the substrate of PLC, PIP_2, PITP was found to be necessary for sustained PLC signalling in reconstitution assays using streptolysin O permeabilised cells. Presumably, PITP allows refilling of the pool of PIP_2 by presenting PI to PI4-kinase. It has also been suggested that PITP potentiates phospholipase D (PLD) activity by increasing the cellular content of PIP_2, which is a putative PLD cofactor (Liskovitch & Cantley 1995).

Figure 3

Phosphatidylinositol-bisphosphate (PIP_2) is hydrolysed by a PIP_2 -specific phospholipase C to generate two second messengers.



The two second messengers generated are:

(i) **Inositol trisphosphate (IP_3)** -a water soluble product released into the cytosol to mobilise intracellular stores of calcium.

(ii) **Diacylglycerol (DAG)**-a membrane anchored lipid second messenger which activates protein kinase C (PKC).

Figure 4

Phospholipases C

1. **Genes:** several purified. 16 amino acid sequences deduced from cDNA (14 mammalian plus 2 drosophila). 10 genes.

2. **3 groups:** β, γ, δ – overal similarity is low exept for two regions i.e. X = 170 aa (60% homology) and Y = 260 aa (40%).

   5 β isoforms identified. 4 mammalian. β1 150 kDa.

   2 mammalian γ isoforms – γ1 145 kDa.

   4 mammalian δ isoforms – δ1 85 kDa.

3. **Structure:** all PLCs have an amino terminal 300 aa region before X domain. β and δ only only 50–70 aa between X and Y. γ = 400 aa region between X and Y which contains the SH2 and SH3 domains.

   All mammalian isoforms have a pleckstrin homology (PH) domain.

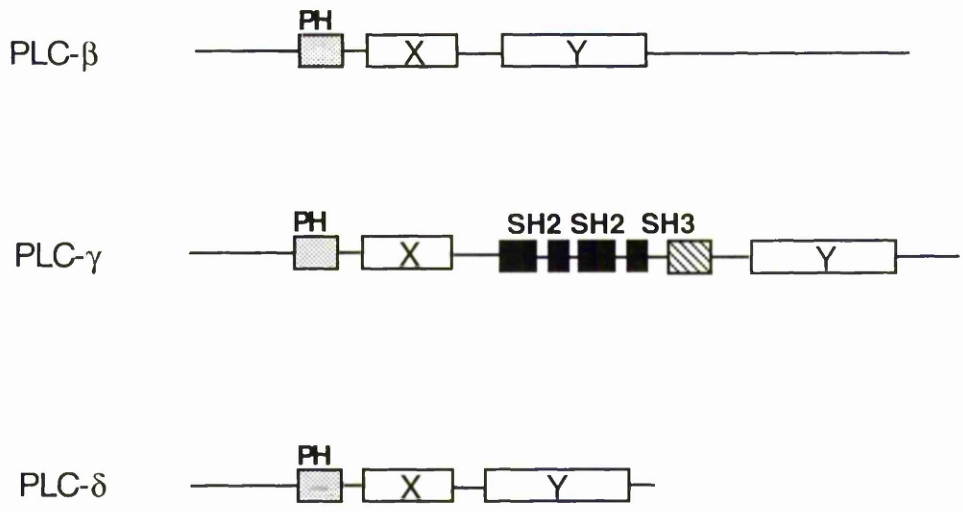
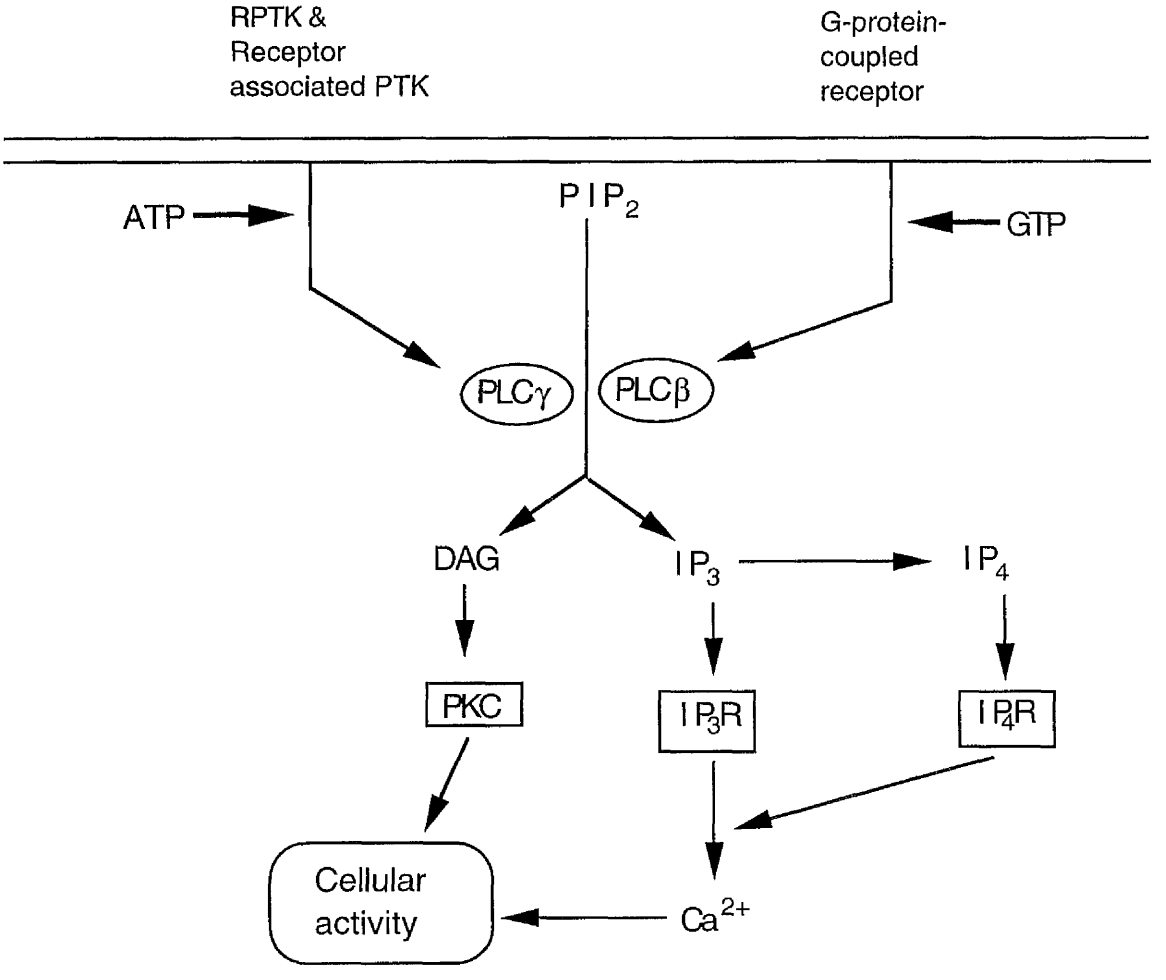


Figure 5

Schematic representation of receptor-protein tyrosine kinase (RPTK), receptor-associaed protein tyrosine kinase, and by G-protein coupled receptor induced phospholipase C (PLC) activation and signal transduction.



1.3.2 Phospholipase D

Phospholipase D (PLD) catalyses the hydrolysis of phospholipids, usually phosphatidylcholine, to generate phosphatidic acid plus the head group. Phosphatidic acid may act directly as a signalling molecule (Moolenaar *et al* 1986, Lambeth *et al* 1994) or can be further metabolized to form diacylglycerol by phosphatidic acid phosphohydrolise (Perry *et al* 1993, Liscovitch & Cantley 1995, Exton 1994). (Figure 6).

1.3.2.1 Classes of PLDs

Two biochemically distinguishable PLD activities have been characterised: one is dependent upon small GTPase Arf and upon PIP_2 (Brown *et al* 1995, Singer *et al* 1995), and another is stimulated by oleate (Okamura & Yamashita 1994). Recently, a gene required for meiosis in *Saccaromyces cerevisia* (Rose *et al* 1996) was isolated and shown to have sequence homology to a castor bean PLD (Wang & Zheng 1994). The first cloned mammalian homologe of the plant PLD, human PLD1 (hPLD1), is regulated by PIP_2, ARF, RhoA, and PKC (Hammond *et al* 1997, 1995). A rat PLD1 (rPLD1) has also recently been cloned and caracterised (Park *et al* 1997) and shows similar properties to hPLD1. Two splice variants of PLD1 have been identified (Hammond *et al* 1997), but no significant differences in activity or in *vitro* regulation of the two forms have been observed. A second isoform of PLD, PLD2, was recently cloned from mouse (mPLD2) (Colley *et al* 1997) and rat (rPLD2) (Kodaki & Yamashita 1997). Both rodent PLD2s require PIP_2 for activity and, unlike hPLD, are constituvely active in the absence of other activating factors when PIP_2 is present. More recently a human PLD2 (hPLD2) has been cloned and partially characterised (Lopez *et al* 1998). Unenexpectedly, hPLD2 is activated not only by PIP_2 but also by ARF-1. Although the magnitude of the activation of hPLD2 by ARF is smaller than of hPLD1. (Figure 7).

1.3.2.2 Mechanisms for PLD activation

It has been suggested that PLD activation can occur through the action of PTKs, PKCs, and small GTPases. (Figure 8).

However the mechanisms of activation of PLD by PTKs remains unclear since most of the information has been obtained with the use of PTKs inhibitors, and it is not known whether PTK activation results in direct phosphorylation of PLD or whether phosphorylation of other intermediate proteins regulate PLD activation (reviewed in Liscovitch & Chafifa-Caspi 1996, and Natarajan et al 1996). (Figure 8).

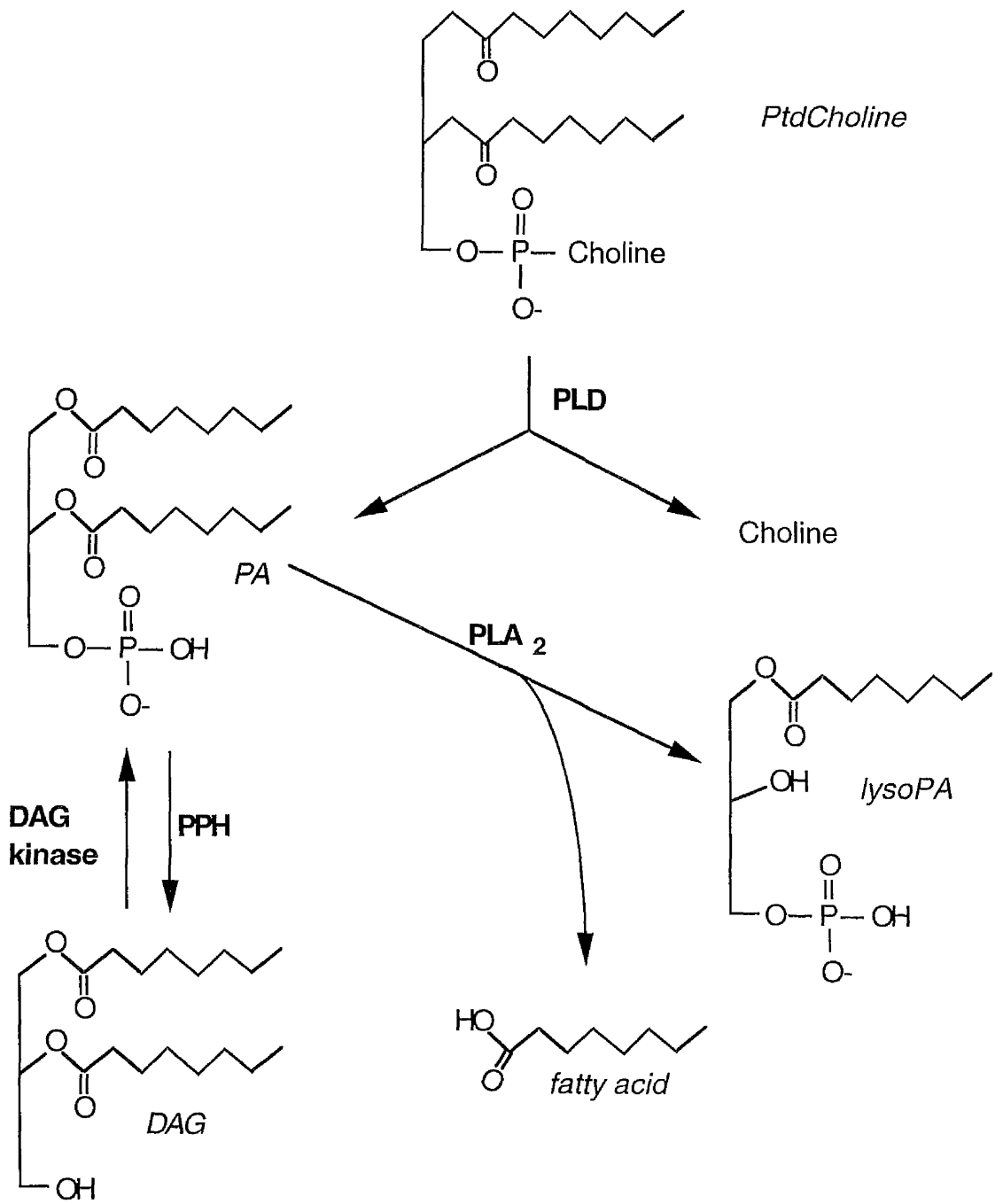
Regulation of phospholipase D by PKC has been shown to require ATP (Lopez et al 1995, Kiss et al 1991). Furthermore, in a study by Conrade *et al* (1994), regulation of a PtdCho-specific PLD activity by PKC was examined in membranes from Chinese hamster lung fibroblasts by using the purified conventional isoenzymes α, β and γ, and the recombinant δ, ε and ζ isoenzymes. Both PKC α and PKC β (in that order) enhanced, while other isoforms failed to modify PLD-mediated PtdCho hydrolysis. Interestingly, in these membranes stimulation of PLD activity by PMA did not require ATP. Collectively, These findings appear to indicate that regulation of PLD activity by PKC can occur by both phosphorylating and non-phosphorylating mechanisms. (Figure 8).

PLD is activated by *v-src* and depends upon a GTPase cascade containing Ras and Ral (Jaing *et al* 1995a & 1995b). Ral constitutively associates with PLD through Ral's novel amino terminus, but Ral does not activate PLD alone (Jiang *et al* 1995b). Although it is not known whether Ral-associated PLD depends upon Arf, as the Arf-dependent PLD does, the Ral-associated PLD activity does depend upon PIP_2 (Jiang *et al* 1995b). Evidence also implicates Rho in PLD activation (Bowman *et al* 1993, Malcolm *et al* 1994). This growing

evidence suggests that PLD activation depends upon a complex interplay of multiple small GTPases. (Figure 8).

Figure 6

PLD hydrolysis of phosphatidylcholine and the generation of lipid second messengers



Phospholypase D (PLD) hydrolyses phosphatidylcholine (PtdCholine) to generate phosphatidic acid (PA). PA can be further hydrolyse to generate lysophophatidic acid (lysoPA) via a cytosolic phospholipase A (PLA 2); or via a phosphatidyl- phosphohydrolase (PPH) to generate diacylglycerol (DAG), this latter step can be reversed via a DAG kinase.

Figure 7

Phospholipases D

1. Genes: very few purified. One plant; one yeast; and one human. However, GenBank blast searches with the human gene sequence identify as many as 13 related sequences in eight prokaryotes and eukaryotes organisms.

2. Three different genes: human PLD (hPLD); yeast PLD (Spo14); plant PLD (*Ricinus* PLD) – overal similarity is low exept for four regions (I; II; III; IV).

hPLD 124 kDa (membrane associated, dependent on PIP_2, PC specific)

Spo14 195 kDa (membrane associated, dependent on PIP_2, PC specific)

Ricinus PLD 92 kDa.

3. Structure: within the four similar regions (I; II; III; IV), homology between the yeast and human enzymes is higher than with cognate secuences of the plant PLD.

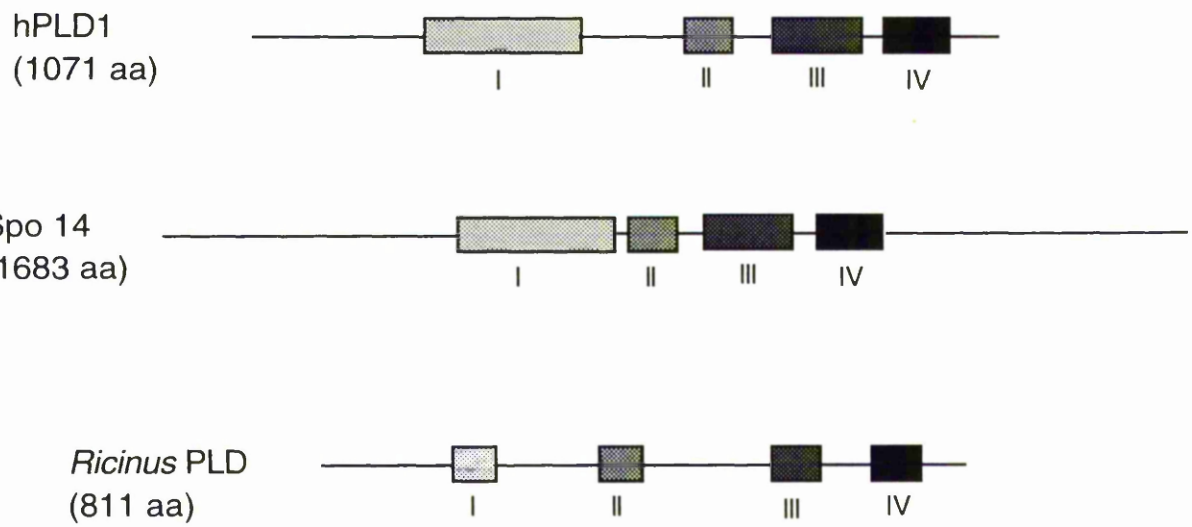
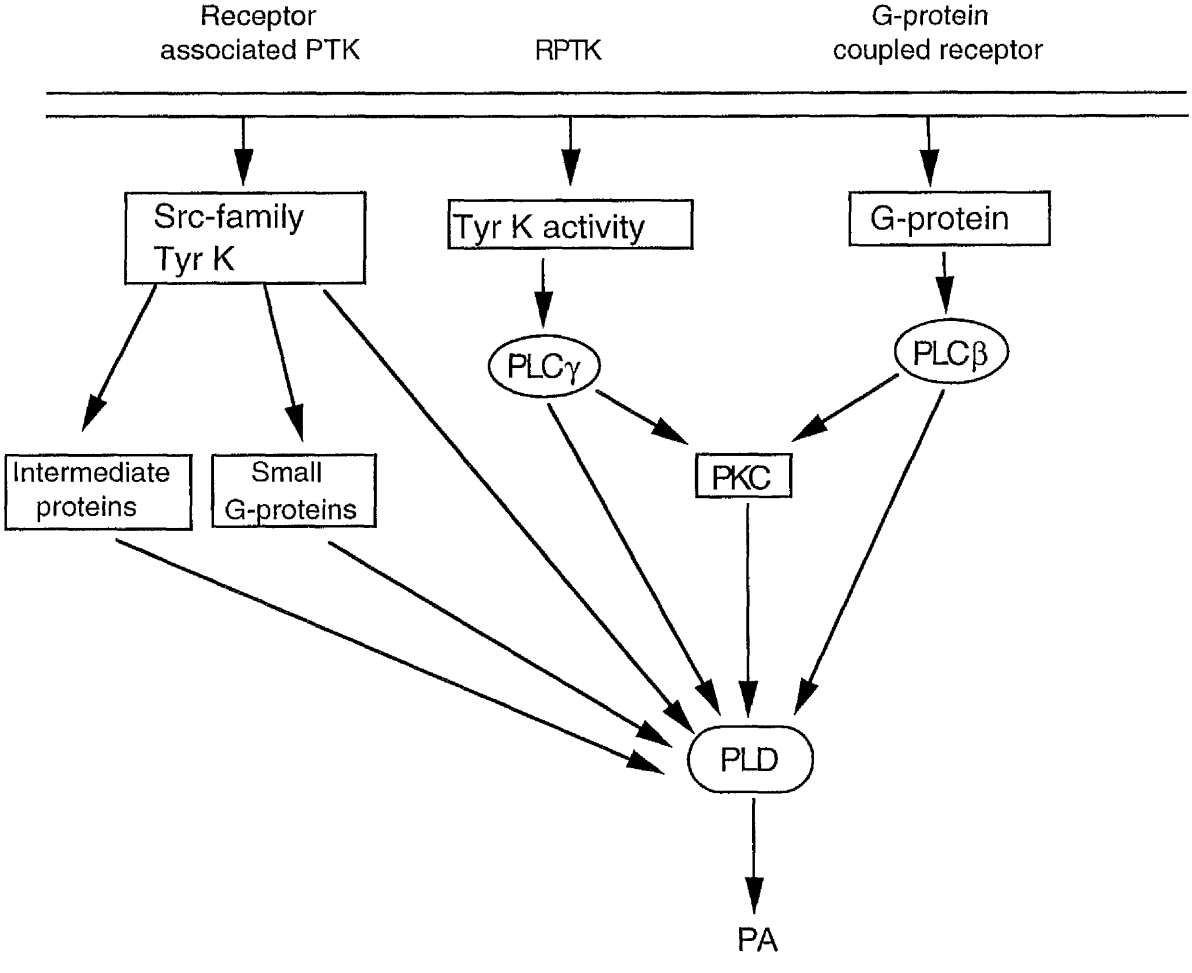


Figure 8

Schematic representation of receptor-protein tyrosine kinase (RPTK), receptor-associaed protein tyrosine kinase, and by G-protein coupled receptor induced phospholipase D (PLD) activation and signal transduction.



1.3.2.3 Second messergers derived from PLD activity

Phosphatidic acid (PA)

Phosphatidic acid (PA) has been implicated as a lipid second messenger in the regulation of protein kinases, GTPase-activating proteins, PI kinases, adenylyl cyclase and other signalling molecules (Foster 1993), however, direct effects of PA have not been clearly established and many effects are probably mediated by the PA metabolites, DAG and lysophosphatidic acid (LPA) (Nishizuka 1992, Moolenaar 1995).

PLD has also been implicated in regulating membrane trafficking and vesicular transport (Brown *et al* 1993, Cockroft *et al* 1994, Kahn *et al* 1993), in which processes acidic phospholipids may facilitate membrane budding and/or fusion (Randazzo & Kahn 1994). (Figure 9).

Lysophosphatidic acid (LPA)

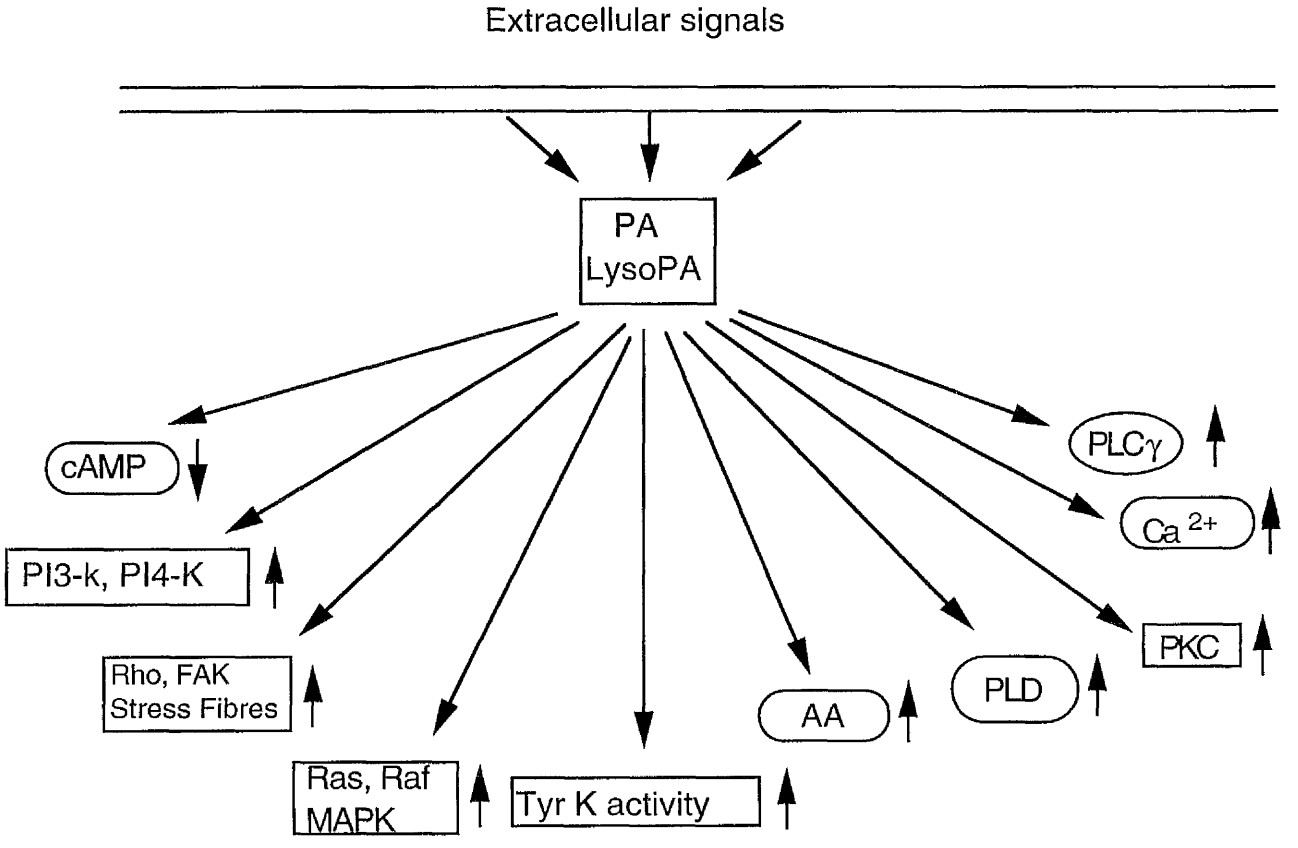
Recently, second messenger function has been ascribed to LPA, which is released from activated cells, especially platelets, and elicits diverse biological responses in different systems including platelet aggregation, cell growth and neurite retraction (Moolenaar 1995, Jalink *et al* 1994). LPA may also play a role in inflammatory and proliferative responses to injury (Moolenaar 1995). (Figure 9).

Mechanism for lysophosphatidic acid action

LPA binds to a 38-40 kDa receptor which couples, via distinct G proteins, to multiple independent effector pathways. The α subunit of Gi directly inhibits adenylyl cyclase, whereas the $\beta\gamma$ dimer is thought to activate Ras via an intermediary protein tyrosine kinase. The LPA receptor, via a mechanism involving Gq and stimulation of PLC, also activates Rho, resulting in stimulation of focal adhesion kinase and phosphorylation and reorganisation of cytoskeletal proteins (Hordijk *et al* 1994).

Figure 9.

Some signalling properties of phosphatidic acid and lysophosphatidic acid.
Many ofthe effects of phosphatidic acid and lysophosphatidic acid are very similar.



Phosphatidic acid (PA) and its metabolite, lysophosphatidic acid (lysoPA), have potent mitogenic effects in several cell lines. They stimulate the respiratory burst in neutrophils; activate phospholipase C gamma (PLCγ); phosphoinositide 4-kinase (PI4-k); phosphoinositide 3-kinase (PI3-k); tyrosine kinases (Tyr K); the Ras-Raf-mitogen activated protein kinase (MAPK) pathway; focal adhesion kinase (FAK); arachidonic acid (AA) production; calcium mobilisation; phospholipase D (PLD) activation; and decrease cyclic AMP (cAMP).

Diacylglycerol (DAG)

A great number of receptor-signalling systems induce an increase in the mass of diacylglycerols (collectively diacylglycerol, alkyl-acylglycerol and alkenyl-acylglycerol; DRG), in particular sn-1,2-diacylglycerol (DAG), the physiological activator of protein kinase C (PKC) (Divecha & Irvine 1995). DAG can be generated by the action of PLC-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate, this DAG generation has been shown to be rapid but transient (Wright *et al* 1988, Cook *et al* 1990). However, a more sustained phase of DAG generation has been associated with an increase in the activation of PLD-catalysed phosphatidylcholine hydrolysis, producing PA which can be converted to DAG by the action of phosphatidic acid phosphohydrolase. It has also been proposed that DAG can also be generated through a PC-PLC pathway, although the evidence for stimulation of this pathway in mammalian cells remains mostly circumstantial (Cook & Wakelam 1992, Exton 1994). It has recently been demonstrated that the DAG and PA derived from PLC activation is predominantly polyunsaturated while the lipids generated by PLD activation are saturated or monosaturated (Pettitt *et al* 1997).

DAG generated through PLD: mixed feelings about signalling

The role of DAG generated through PLD as a second messenger, and indeed in activating PKCs is still controversial. It has been demonstrated that PA, generated by the activation of PLD, can stimulate rho-mediated actin stress fibre formation in porcine aortic endothelial (PAE) cells (Cross *et al* 1996). This would suggest that, while PLD activation clearly results in an increase in DAG, it is not certain that this lipid plays a signalling role. Rather, it may be that the initial product, PA, functions as a messenger and that it is converted to DAG to attenuate the signal.

The *in vitro* and *in vivo* experiences

In vitro studies have demonstrated that essentially all DAG species can activate PKC, although the *in vivo* evidence for PKC activation by PLD-derived DAG remains mixed. It has been shown there is no activation of PKC in IIC9 fibroblasts when stimulated with a concentration of thrombin, which did not activate inositol lipid hydrolysis but did activate PLD (Leach *et al* 1991). More recently it has been shown that PLD-derived DAG does not appear to regulate PKC activity in stimulated PAE cells (Pettitt *et al* 1997). On the other hand, it has been found that PKCε is activated under the same conditions (Ha & Exton 1993).

1.3.3 Phosphoinositide 3-kinases and the generation of lipid second messengers

Phosphoinositide 3-kinases (PI3-kinases) catalyse the phosphorylation of inositol phospholipids at the 3-position of the inositol ring (Whitman *et al* 1988). The phospholipids produced by the actions of these enzymes; PtdIns(3)P, PtdIns(3,4)P_2 (PIP_2) and PtdIns(3,4,5)P_3 (PIP_3), act as second messengers which are increasingly implicated in regulating a number of cellular responses. For example, PI3-kinase activity has been implicated as being involved in insulin-induced glucose transport (Okada *et al* 1994), platelet-derived growth factor- and insulin-induced actin rearrangement (Wymann & Arcaro 1994, Kotani *et al* 1994) and in the regulation of neuronal survival (Dudek *et al* 1997).

1.3.3.1 Classes of PI3-kinases

There are three classes of PI3-kinases which are determined by their *in vitro* substrate specificities. Class I PI3-kinases phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2. This class of enzymes can be further subdivided into class IA

and class IB. Class IA PI3-kinases consist of p110 catalytic subunits that are regulated by the SH2/SH3-domain containing p85 family of adaptor proteins and consist of the isoforms p110α, p110β, and p110δ (Hiles *et al* 1992, Hu *et al* 1993, Vanhaesebroeck *et al* 1997). The p85 family of adaptor proteins (p55α, p55 PIK, p85α, and p85β) (Otsu *et al* 1991, Pons *et al* 1995, Inukai *et al* 1996) facilitate PI3-kinase interactions with other proteins through their SH2 and SH3 domains. Class IB consists of p110γ, an enzyme which associates with a p101 adaptor protein and is stimulated by G-protein βγ subunits (Stephens *et al* 1994, Stoyanov *et al* 1995, Stephens *et al* 1997, Tang & Downes 1997). Class II PI3-kinases phosphorylate PtdIns and PtdIns(4)P but not PtdIns(4,5)P_2 (MacDougall *et al* 1996, Molz *et at* 1996) and class III PI3-kinases have a substrate specificity restricted to PtdIns and are homologous to yeast Vps34p (vacuolar protein sorting defective) which is involved in the trafficking of proteins from the Golgi to the yeast vacuole (Hernan *et al* 1991, Stack & Emr 1995, Volinia *et al* 1995).

Class 1A and 1B are differentially activated

Previous studies have shown that receptors that are coupled to tyrosine kinases, such as insulin, exclusively activate class IA PI3-kinases through the p85 adaptor molecule (Hiles *et al* 1992, Hu *et al* 1993, Vanhaesebroeck *et al* 1997). In contrast, receptors that engage heterotrimeric G proteins, such as ATP, exclusively activate class IB PI3-kinases through the generation of βγ subunits (Stephens *et al* 1994, Stoyanov *et al* 1995, Stephens *et al* 1997, Tang & Downes 1997). No crosstalk between the coupling of these two receptor to PI3-kinases has been shown.

1.3.4 Sphingolipid-derived second messengers

Hydrolysis of sphingomyelin, catalysed by sphingomyelinase, a sphingomyelin-specific PLC, yields ceramide and phosphorylcholine.

Cleavage of the amide-linked fatty acid from ceramide to form sphingosine is catalysed by ceramidases. Sphingosine can be metabolised further, via a pathway involving sphingosine kinase catalysed phosphorylation, to sphingosine 1-phosphate (SPP), and SPP can be cleaved to ethanolamine phosphate and *trans*-2-hexadecanal by a specific lyase located in the endoplasmic reticulum (reviewed in Spigel *et al* 1996). (Figure 10).

1.3.4.1 Ceramide as a second messenger

A number of direct targets for ceramide action have now been identified; including a ceramide-activated protein kinase (CAPK) (Liu *et al* 1994), a ceramide-activated protein phosphatase (CAPP) (Dobrowsky & Hannun 1992), the putative guanine-nucleotide exchange factor Vav (Gulbins *et al* 1994) and the ζ isoform of protein kinase C (Lozano *et al* 1994, Muller *et al* 1995).

The best known ceramide second messenger function is derived from studies on TNFα induced inflammation and apoptosis. Ceramide appears to be a primary mediator of both the inflammatory and the apoptotic responses to TNFα.

Ceramide and inflammation

With respect to the inflammatory response, ceramide stimulates CAPK to phosphorylate Raf 1, enhancing Raf 1 activity. This initiates signalling through the MAPK cascade (Yao *et al* 1995), and results in activation of cytosolic phospholipase A_2 and release of arachidonic acid.

Ceramide and apoptosis

With regards to apoptosis, TNFα induces rapid hydrolysis of sphingomyelin to ceramide in all cells in which it initiates apoptosis. Ceramide also signals Fas-mediated apoptosis (Cifone *et al* 1993, Gulbins *et al* 1995). Ceramide-mediated apoptosis extends beyond apoptosis induced by cytokine receptors: ionising radiation appears to directly target membranes, resulting in

hydrolysis of sphingomyelin to ceramide within seconds (Haimovitz-Friedman *et al* 1994). This latter event may initiate an interphase form of apoptotic death.

1.3.4.2 Sphingosine and SPP as second messengers

Sphingosine and SPP were originally proposed as negative regulators of PKC (Hannun & Bell 1989). However, more recently it has been shown that sphingosine and SPP play different roles in signalling. In this regard, sphingosine induced a mitogenic effect that was stereospecific and independent of PKC inhibition in 3T3 fibroblasts (Zhang *et al* 1990, Olivera *et al* 1994).

Mitogenic effects of Sphingosine and SPP

Sphingosine and SPP may also regulate T-cell proliferation (Olivera & Spiegel 1993). Growth inhibition by ISP-1/myriocin, a potent immunosuppressant which inhibits serine palmitoyltransferase and consequently sphingoid base synthesis, was reversed by exogenous sphingosine or SPP (Miyake *et al* 1995). Furthermore, certain growth factors, such as PDGF, induced a rapid, transient elevation in sphingosine and SPP levels in fibroblasts (Olivera & Spiegel 1993) in arterial smooth muscle cells (Bornfeldt *et al* 1995) and in glomerular mesangial cells (Coroneos *et al* 1995). Furthermore a pharmacological inhibition of the generation of these sphingolipids reduced PDGF-induced cellular proliferation (Olivera & Spiegel 1993, Bornfeldt et al 1995, Coroneos *et al* 1995).

Sphingosine as inhibitor of cell growth

Although the mitogenc effects of sphingosine and SPP appear widespread, in some cell types sphingosine inhibits cell growth, perhaps via PKC inhibition (Hannun & Bell 1989). Sphingosine may even be an endogenous mediator of

apoptosis (Ohta *et al* 1995), although its acylation to produce ceramide in most systems confounds this issue.

Sphingosine and SPP in calcium mobilisation.

Sphingosine and SPP mobilise calcium from internal stores via an inositol-trisphosphate-independent pathway (Mattie *et al* 1994, Ghosh *et al* 1994). The response of calcium to SPP has many hallmarks of a receptor-mediated event, including rapidity, reversibility, and specificity. As the endoplasmic reticulum contains the kinase which converts sphingosine to SPP, all the elements sufficient for regulated calcium signalling via these lipids appear to be topologically compartamentalised (Ghosh *et al* 1994). The electrophysiological and biophysical properties of a sphingolipid-gated intracellular calcium-permeable channel has previously been characterised (Kidman *et al* 1994).

A receptor for sphingolipid mediated-intracellular-calcium release.

The molecular cloning and characterisation of a protein that causes the induction of sphingosyl-phosphocholine-mediated calcium release has been reported: this is known as SCaMPER (for **s**phingolipid **c**alcium release-**m**ediating **p**rotein of **e**ndoplasmic **r**eticulum) (Mao *et al* 1996). The properties of SCaMPER are identical to those of the sphingolipid-gated calcium-permeable channel that was previously described (Kidman *et al* 1994, Mao *et al* 1996). These findings suggest that SCaMPER is a sphingolipid-gated calcium-permeable channel and supports its role as a mediator of this pathway for intracellular calcium signal transduction.

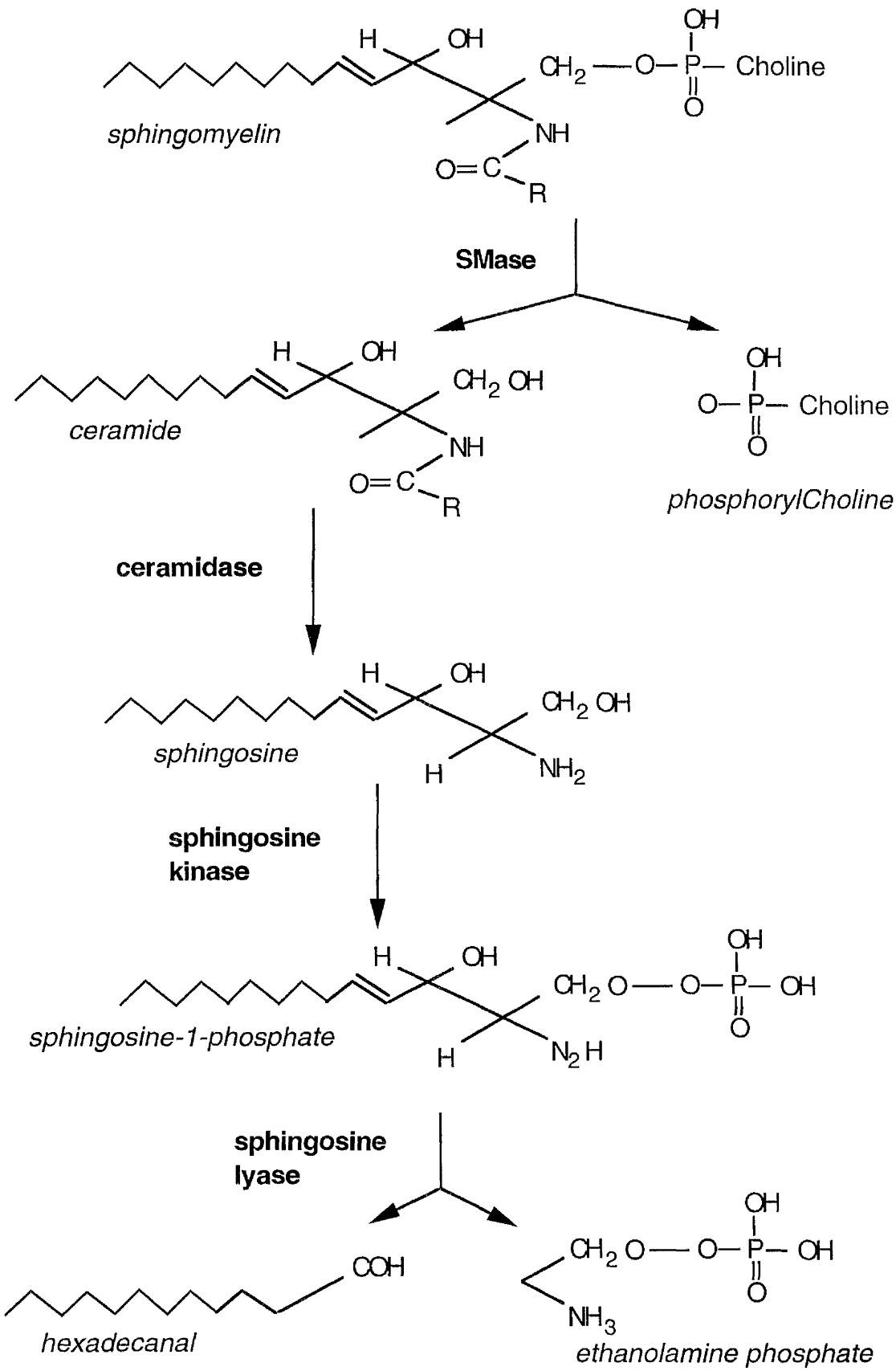
A cell surface receptor for SPP.

Although many studies indicate an intracellular site of action of SPP, some of its biological effects when added exogenously, such as inhibition of motility and invasiveness, may be due to extracellular effects on cell surface receptors (Yatomi *et al* 1995). SPP is stored in high concentrations in human platelets,

is released upon activation by physiological stimuli, and may play a role in platelet aggregation (Yatomi et al 1995). Gi/Go-coupled receptors may be directly or indirectly involved, as some responses to SPP are pertusis toxin sensitive. Furthermore, very recently it was shown that SPP is a ligand for the G-protein-couped orphan receptor EDG-1 (Lee et al 1998).

Figure 10

Sphingolipid-derived second messengers



1.4 SIGNALING BY THE Ser/Thr PROTEIN KINASE C (PKC)

Serine/threonine protein kinases C (PKCs) are intracellular kinases involved in almost all intracellular signalling events.

1.4.1 Different PKCs

Protein kinases C (PKC) is a family of serine/threonine protein kinases which share a common requirement for phospholipid. PKC isoenzymes, depending on their structure and cofactor regulation, are divided into three groups (Hug & Sarre 1993, Dekker & Parker 1994): conventional (cPKC α; β1; β2; and γ) which are calcium and DAG activated isoenzymes, novel (nPKC δ; ε; η; and θ) which are calcium independent but DAG activated isoenzymes, and the atypical ones (aPKC ζ; λ/ι; and μ) which do not require either calcium or diacylglycerol (Hug & Sarre 1993, Dekker & Parker 1994, Nishizuka 1988 & 1989, Kazaniets *et al* 1993). (Figure 11).

1.4.2 Mechanisms for PKC activation

Recent investigations have shown that structural elements of PKC mediate lipid activation (Newton 1995). The lipid binding moieties of cPKCs have long been known to reside in two conserved modular domains (C1 and C2). Resolution of the crystal structure of the second cysteine-rich region of the C1 domain of PKCγ bound to phorbol ester revealed that the phorbol ester binding site is formed by β sheets, contained in the top half of the C1 domain, that are pulled apart to form a cavity (Zhang *et al* 1995). Insertion of phorbol ester into this site does not alter the conformation of this domain but rather results in a surface that is continuously hydrophobic, perhaps allowing for efficient interactions with membranes. The lower half of the domain contains the metal-binding sites and appears to be responsible for maintaining the appropriate conformation of the domain. PKC isoenzymes

that contain C1 regions but do not bind phorbol ester lack a particular conserved proline residue (Newton 1995).

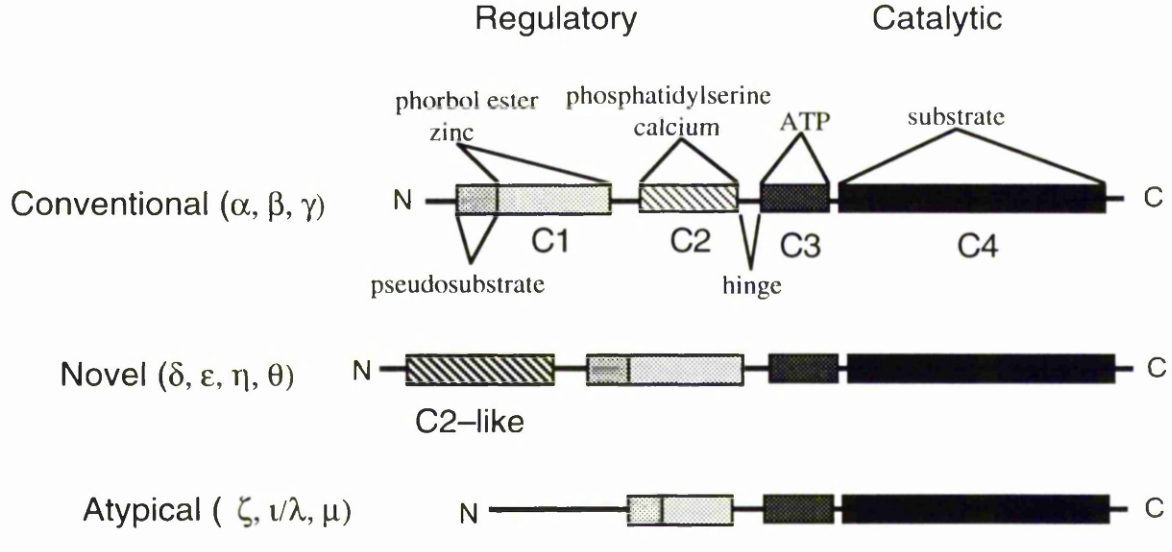
Figure 11

Schematic representation of the primary structure of conventional, novel, and atypical protein kinase Cs

Protein kinase C isoenzymes are single polypeptides, comprised of an N–terminal regulatory region (20–40 kDa) and a C–terminal catalytic region. Four conserved domains (C1–C4) are found in most PKCs.

C1 contains a Cys–rich motif that forms the diacylglycerol/phorbol ester binding site, this domain is immediately preceded by an autoinhibitory pseudosubstrate sequence.
C2 contains the recognition site for acidic lipids and, in some isoenzymes, the calcium–binding site.
C3 contains the ATP–binding lobe of the kinase core.
C4 contains the substrate–binding lobe of the kinase core.

The regulatory and catalytic halves are separated by a hinge region that becomes proteolytically labile when the enzyme is activated.



1.5 SIGNALLING THROUGH FcγRI

Human FcγRI, a high-affinity IgG receptor, is an integral type I transmembrane glycoprotein (Allen & Seed 1989) constitutively expressed on monocyte and macrophage cell types. The cytoplasmic tail of FcγRI contains no obvious signalling motif or homology with other immune system receptors. Aggregation of FcγRI, however, results in signal transduction, seen as protein tyrosine phosphorylation events (Liao *et al* 1992, Scholl *et al* 1992, Rankin *et al* 1993, Lin *et al* 1994) and tyrosine kinase dependent calcium transients (Davis *et al* 1994, van de Winkel *et al* 1990). In myeloid cells, FcγRI is associated non-covalently with the γ chain protein (Ernst *et al* 1993, Scholl & Geha 1993), which contains an ITAM necessary for activation of src–like and syk kinases (Reth 1989, Samuelson & Klausner 1992, Cambier 1995). (Figure 2).

One feature of monocytes and macrophages is the heterogeneity of response to immune complex challenge using cells harvested under different conditions and different environments. Aggregation of Fcγ receptors lead to the tyrosine phosphorylation of phospholipase Cγ1 (Liao *et al* 1992) and resulting cytosolic calcium transients (Rankin *et al* 1993). Little is known, however, about the signal transduction mechanisms underlying FcγRI signalling, or how these mechanisms are modified as blood monocytes differentiate into tissue macrophages. Thus, to study early events in the FcγRI signalling pathway, we have used the human monocyte cell line, U937 (Harris & Ralph 1985), which constitutively expresses FcγRI and FcγRII and which allows controlled differentiation into a more macrophage cell type by treatment with dibutyryl cAMP (dbcAMP) (Sheth *et al* 1988). Previous work has shown that the nature of calcium transients markedly changes as the cells become differentiated (Davis *et al* 1994). Thus, a single spike in calcium is

observed in response to Fcγ receptor aggregation of cells treated with interferon-γ whereas calcium oscillations are generated in cells differentiated to a more macrophage state by dbcAMP (Floto *et al* 1995).

1.6 AIMS OF THE PRESENT STUDY

The aims of this study were to investigate the signal transduction mechanisms underlying FcγRI signalling and how these mechanisms are modified as blood monocytes differentiate into tissue macrophages using the human monocyte cell line, U937.

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CHAPTER 2

MOLECULAR SWITCH BY FcγRI ANTIBODY RECEPTOR MOBILISES CALCIUM THROUGH TWO DISTINCT SIGNALLING PATHWAYS

2.1 ABSTRACT

Background: Aggregation of receptors for the Fc region of IgG activates a repertoire of responses that can lead to targeted cell killing via antibody directed cellular cytotoxicity (ADCC). The nature of the myeloid cellular response to immune complexes is highly variable and depends on the maturation state of the cell.

Results: We show here that differentiation of a monocytic cell line, U937, to a more macrophage phenotype results in an absolute and fundamental switch in the nature of the phospholipid signalling pathway recruited following Fcγ receptor aggregation. In cytokine primed cells, FcγRI aggregation results in the activation of phospholipase D and sphingosine kinase whereas no products of phospholipase C activation could be detected. In addition, release of stored calcium was secondary to phospholipase D and sphingosine kinase activation. In contrast, in cells differentiated to a more macrophage type, aggregation of FcγRI resulted in activation of phospholipase C and neither sphingosine kinase nor phospholipase D was activated. Moreover, the resulting calcium transients were prolonged as calcium entry was stimulated. Thus, differentiation switches the nature of phospholipase recruitment by FcγRI and this switch dictates the type and duration of calcium transients and hence, provides a molecular mechanism for the distinct differentiation-dependent responses activated as cells mature to a more macrophage-like cell.

Conclusions: Our data demonstrates that the switch in signalling pathways is defined by a switch in accessory molecule recruitment by FcγRI as this receptor lacks an intrinsic tyrosine activation motif. As many immune receptors display ligand binding and signal transduction on separate chains (thereby opening the possibility of a switch in signal transduction) the mechanism defined here is therefore likely to have wide applications.

2.2 INTRODUCTION

Binding of the constant region (Fc) of immunoglobulin G (IgG) molecules to its surface Immune Recognition Receptor (FcγR) of leukocytes provides a pivotal link between the humoral and cellular arms of the immune system [see reviews 1-3]. On myeloid cells, aggregation of these receptors triggers a number of different effector functions including endocytosis of immune complexes or phagocytosis of opsonised particles. Fcγ receptor aggregation activates a repertoire of responses including degranulation and release of proteases, activation of the respiratory burst and release of cytokines. These can ultimately lead to targeted cell killing through antibody directed cellular cytotoxicity (ADCC) [4, 5] which is critically important for clearing virus infected cells and in cancer surveillance [6]. One feature of monocytes and macrophages is the heterogeneity of responses to immune complex challenge using cells harvested under different conditions and different environments.

Three different classes of FcγR have been defined; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Of these, the human high affinity receptor, FcγRI, is an integral type I membrane glycoprotein [7] constitutively expressed on monocyte and macrophage cell types. The cytoplasmic tail of FcγRI contains no obvious signalling motif or homology with other immune system receptors. However, aggregation of FcγRI results in signal transduction as evidenced by protein tyrosine phosphorylation events [8-11] and tyrosine kinase dependent calcium transients [12, 13]. In myeloid cells, FcγRI is associated non-covalently with the γ chain [14, 15] which contains an immunoreceptor tyrosine activation motif (ITAM) necessary for activation of soluble tyrosine kinases [16-18]. The γ chain, a 7 kDa protein originally identified as a component of the high affinity IgE receptor (FcεRI) in mast cells [19], is a member of a family of signal transducing molecules that includes the

ζ chain of the T cell receptor [20]. As a result of the non-covalent association between FcγRI and the γ chain, receptor aggregation results in clustering of the associated γ chain ITAMs and thereby triggers tyrosine kinase recruitment and activation [21-23].

Activation of IgG-loaded receptors leads to the tyrosine phosphorylation of phospholipase Cγ1 [8] and resulting cytosolic calcium transients [10]. However, little is known about the signal transduction mechanisms underlying FcγRI signalling or how they are modified as blood monocytes differentiate into tissue macrophages. Thus, to study early events in the FcγRI signalling pathway, we have used the human monocyte cell line, U937 [24], which constitutively expresses FcγRI and FcγRIIa and which allows controlled differentiation into a more macrophage cell type by treatment with dibutyryl cAMP (dbcAMP) [25]. Previous work has shown the nature of the calcium transients markedly change as the cells become differentiated [12]. Thus, a single spike in calcium is observed in response to FcγR aggregation of cells treated with interferon-γ whereas calcium oscillations are generated in cells differentiated to a more macrophage state by dbcAMP [26]. We now show that there is a fundamental and absolute developmental switch in the particular phospholipase pathway recruited following Fcγ receptor aggregation during differentiation from a monocyte to a macrophage-like cell and that the types of calcium transients generated intracellularly are consequent on this switch in signalling pathway.

2.3 MATERIALS AND METHODS

2.3.1 Materials and Cell Culture

U937 cells were cultured in RPMI1640 (Gibco) supplemented with foetal calf serum (10%), glutamine (2 mM, penicillin (10U/ml) and streptomycin (10μg/ml) at 37°C, 6.8% carbon dioxide in a water saturated atmosphere. The cells were treated with IFN-γ (Bender Wein Ltd) (200ng/ml) for 24 hours or dbcAMP (1mM) for 48 hours.

Antisense oligonucleotides were purchased from Oswell DNA Services (Southampton, UK). 24 mers were synthesised, capped at either end by the phosphothiorate linkages (first two and last two linkages), and correspondedto the reverse complement of the first 8 amino acids for either γ chain or FcγRIIa. The sequences of the oligonucleotides were for γ chain 5' GAG CAA GAC CAC TGC TGG AAT CAT 3', for FcγRIIa 5' TCT GGG ACA TAC ATT CTG AGA CAT 3' and the jumbled control 5' CTG GTG GAA GAA GAG GAC GTC CAT 3'. Cells were incubated in 10μM oligonucleotide for 1 hour prior to, and then for the duration of culture with either IFN-γ or dbcAMP. Addition of DOTAP (Boehringer-Mannheim) was found to have no influence on the effect of the antisense oligonucleotides.

2.3.2 Receptor aggregation for the biochemical assays

Cells were harvested by centrifugation (200g). For experiments using ligand to aggregate the receptors, cells were incubated for 30mins on ice with 1μM human monomeric, polyclonal IgG (Serotec) to occupy the surface FcγRI. Excess unbound ligand was removed by dilution and centrifugation of the cells. Cells were resuspended and ligand occupied receptors were then aggregated by addition of 1:100 dilution of F(ab) goat anti-human IgG (Sigma)

on ice (Figure 4). Cells were then warmed to 37°C for the times specified in the assays.

To aggregate specific Fcγ receptors, cells were incubated with specific monoclonal antibodies for 30 mins on ice (Figure 4). To aggregate FcγRI, cells were incubated with 1 μg of the monoclonal antibodies 22 and 32 which recognise separate epitopes on FcγRI away from the binding pocket. To aggregate specifically FcγRIIa, cells were incubated with the monoclonal antibody, 2e1 (Serotec) in the presence of 3μM human IgG4 (Serotec) to occupy the ligand binding pocket of FcγRI. After removal of excess antibody by dilution and centrifugation, the receptors were crosslinked by the addition of F(ab) goat anti-mouse IgG F(ab) (1:50 dilution) (Sigma). Cells were then warmed to 37°C for the times specified in the assays.

2.3.3 Measurement of Inositol Phosphates

Inositol phosphates were assayed essentially as described in Harnett and Harnett [39]. Briefly, U937 cells were labelled with myo-[^{3}H]-inositol (1μCi/10^6 cells) for 16 hours at 37°C. To measure total accumulated InsP, cells (at 1-3x10^7 cells/ml) were incubated for 10 mins in the presence of 10mM LiCl in RPMI 1640/10mM HEPES/0.1% BSA (RHB) medium, pH7.4 prior to receptor crosslinking and 10mM LiCl was added to all solutions throughout [27, 28]. Following crosslinking of the receptors, the cells were extracted by a Bligh-Dyer phase separation and levels of [^{3}H]inositol trisphosphate ($InsP_3$) or total [^{3}H]inositol phosphates (reaction mixture containing 10 mM LiCl) were determined by liquid scintillation counting of fractions eluted following Dowex (formate form) ion-exchange chromatography of aliquots of the aqueous phase.

2.3.4 Measurement DAG Generation

Mass DAG was measured as described in Briscoe et al [27]. The lower organic phase of Bligh-Dyer extractions were dried *in vacuo* and the lipids solubilised in a Triton X-100/Phosphatidylserine mixture. *E coli* diacylglycerol kinase (Calbiochem) was added to a final concentration of 5 mUnit and the reaction started by addition of 10µl of 5mM ATP containing 1µCi of [^{32}P]-γ-ATP made up in 100mM Imidazole pH6.6. After 30 min., the reaction is stopped by addition of 1ml of chloroform:methanol: HCl (150:300:2) and phases separated by addition of 300µl of chloroform and 400µl of H_2O. Samples of the organic phase were then dried *in vacuo*, solubilised in 40µl of chloroform:methanol (19:1) and resolved on silica TLC plates (Merck, 5714, 5x20cm $60F_{254}$)developed in chloroform: methanol:acetic acid (38:9:4.5). The PtdOH band (relative to standards) was scraped and the associated radioactivity determined by liquid scintillation counting.

2.3.5 Measurement of Phospholipase D Activity

PLD activity was measured by the transphosphatidylation assay [27, 28]. Briefly, U937 cells, were labelled (10^6 cells/ml) with [^{3}H]palmitic acid (5 µCi/ml) in RPMI 1640 medium containing 5% (v/v) foetal calf serum for 16h. Following labelling, the cells were washed in ice cold RPMI 1640/10mM HEPES/0.1% BSA (RHB medium), resuspended at $2x10^6$ cells/ml and incubated at 37°C for 15 mins in RHB medium containing butan-1-ol (0.3% final). Specific Fc receptors were crosslinked as described above and, after the times indicated, cells were extracted by Bligh-Dyer phase separation. Samples of the lower organic phase were resolved on pre-run, heat activated TLC plates (20 x 20 cm, Silica gel 150A grooved plates, Whatman) developed in the organic phase of the solvent, ethyl acetate: 2,2,4 trimethylpentane: acetic acid: water (11:5:2:10). The [^{3}H]PtdBut-containing silica indicated by the

phosphatidylbutanol standard was then scraped into scintillation fluid and counted.

2.3.6 Measurement of sphingosine kinase activity

Sphingosine kinase was assayed as described in Olivera et al, [40]. Reactions were terminated at the times specified in Figure 7 by addition of ice-cold PBS. After centrifugation, the cells were resuspended in ice-cold 0.1M phosphate buffer (pH7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, phosphatase inhibitors (20 mM $ZnCl_2$, 1 mM sodium orthovanadate and 15 mM sodium fluoride), protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF) and 0.5 mM 4-deoxypyridoxine. Cells were disrupted by freeze thawing and centrifuged at 105,000g for 90 min at 4°C. Supernatants were assayed for sphingosine kinase activity using sphingosine (Sigma) and [γ^{32}P]ATP (2μCi, 5mM). as specified in Olivera et al, [40]. After incubation, products were separated by TLC on silica gel G60 using chloroform:methanol:acetic acid: water (90:90:15:6) and visualised by auoradiography. The radioactive spots corresponding to sphingosine phosphate were scraped and counted in a scintillation counter.

2.3.7 Measurement of Cytosolic Calcium

Single cell measurement of changes in cytosolic calcium:

U937 cells treated with dbcAMP (1mM) or IFN-γ (200ng ml^{-1}) were harvested and cells resuspended in HEPES-buffered saline (HBS) supplemented with 1mM $CaCl_2$. Cells were incubated simultaneously with primary antibody (either human human IgG (1μM) or the FcγRI specific mAb 32 or FcγRIIa specific mAb 2e1 plus (1μM) human IgG4) and 5μM Fluo3-AM (Molecular Probes) mixed with an equal volume of 25%w/v Pluronic F127 at room temperature for 30 min. After centrifugation, cells were resuspended in

HBS (1mM Ca^{2+}) at room temperature and aliquots were settled on poly-L-lysine coated coverslips and placed in a perfusion chamber heated to 37°C. The perfusion bath was thoroughly perfused with HBS (2mM Ca^{2+}) to remove excess antibody and ensure complete solution change. For cells loaded with human IgG, receptors were then aggregated by addition of F(ab) fraction of goat anti-human IgG F(ab) antibody (1:50 dilution). For cells loaded with the FcγR specific mAbs, receptors were crosslinked with a F(ab) preparation of a goat antibody specific for mouse IgG F(ab). Scanning confocal imaging was achieved using a Zeiss inverted confocal microscope. Excitation light (488 nm) was provided by an argon laser and emitted light was detected at wavelengths above 515 nm. Laser attenuation, pinhole size and photomultiplier brightness/contrast settings were maintained constant for all experiments. Frames were acquired every 3s (512 x 512 pixel) and pixels assigned intensity values (0-255). Transmitted visible light images were routinely taken before and after every experiment to exclude artefactual changes in fluorescence caused by movement of cells in the focal plane. Results were adjusted for dye photobleach assessed in unstimulated cells. Data analysis were carried out using Metamorph and Sigmaplot (Jandel Scientific Corp, USA) software. Results are expressed as the mean ± standard error. Oscillations were defined as responses with two or more peaks separated by more than one data point.

Measurement of calcium concentrations in cell populations:

To measure the release of calcium from intracellular stores, experiments were performed on cell populations in nominally Ca^{2+} free HBS (at 37°C) using a Cairn Research Spectrophotometer and fura-2 (Molecular Probes) loaded cells as described previously [12]. The backgound corrected 340/380 ratio was calibrated using the method of Grynkiewicz et al [41]. Following each experiment, cells were lysed by the addition of 50μM digitonin in the

presence of external 2 mM Ca^{2+} to give an R_{max} value. R_{min} was subsequently determined by the addition of 20mM EGTA (pH7.4) in the presence of equimolar concentration of Tris base.

2.3.8 Northern analysis

Cells were treated with IFN-γ or dbcAMP and harvested at set times. After centrifugation, the cells were lysed by addition of guanidinium thiocyanate and the RNA was extracted using the acidified phenol method of Chomczynsky and Saachi [42]. The Equal amounts of RNA were electrophoresed through 1% agarose containing formaldehyde, transferred to Nytran membranes (Amersham) and probed sequentially for mRNA to FcγRI [7], FcγRIIa [33] and γ chain [19] using cDNAs for each labelled with ^{32}P by random priming (HiPrime - Boehringer Mannheim). The blots were then probed with tRNA (gift from Dr R. White) labelled in the same way to allow normalisation. The hybridisation signal was visualised by placing against X ray film.

2.4 RESULTS

2.4.1 Section 1: mobilisation of calcium undergoes a differentiation-dependent molecular switch

2.4.1.1 *Differential generation of inositol phosphates following aggregation of Fcγ receptors on differentiated and cytokine treated cells.*

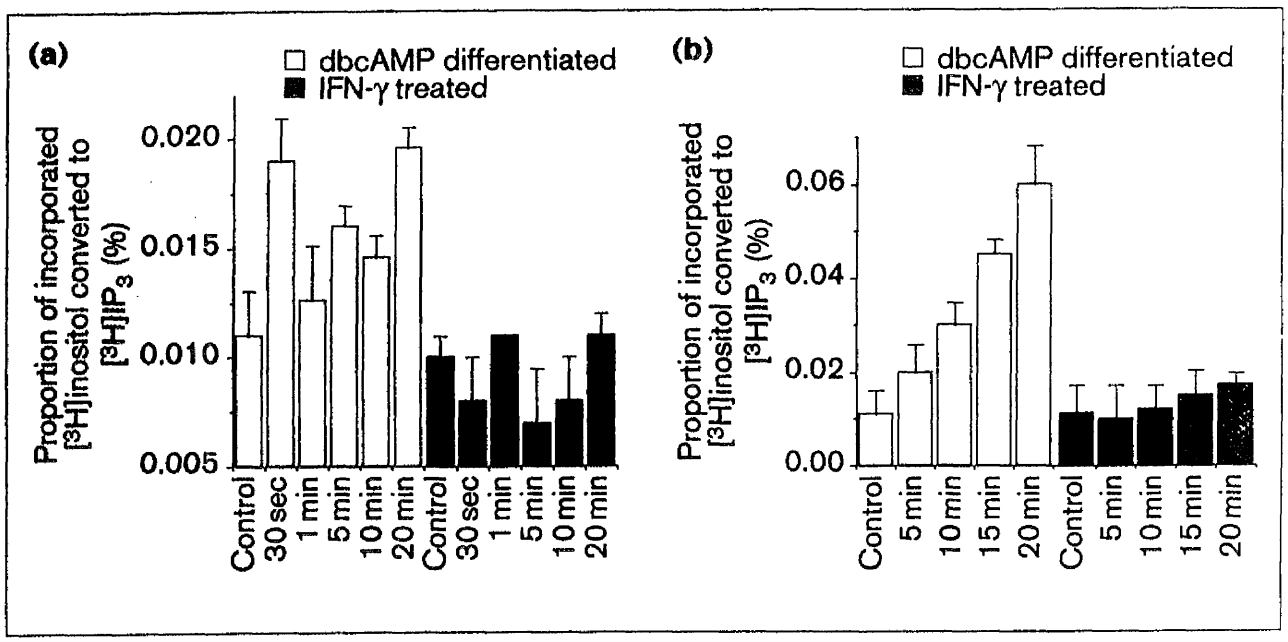
To examine the mechanism underlying the differentiation-dependent change in calcium transients in U937 cells, experiments were performed to define Fcγ receptor coupling to lipid signalling pathways that generate candidate messengers for eliciting calcium release. Thus, as it is well established that aggregation of Fcγ receptors leads to the recruitment of tyrosine kinases and subsequent phosphorylation and presumed activation of $PtdInsP_2$-PLC [8, 10, 11] with resultant generation of the calcium mobilising second messenger, $InsP_3$, our first approach was to investigate coupling to this pathway. Crosslinking of Fcγ receptors on IFN-γ treated and dbcAMP differentiated U937 cells did indeed show differential coupling to $PtdInsP_2$-PLC. A small but significant transient rise in $InsP_3$ was observed in cells differentiated with dbcAMP following receptor aggregation (Figure 1a). By contrast, no increase in $InsP_3$ could be detected following receptor aggregation by the same stimulus in cells treated with IFN-γ (Figure 1a).

As the increase in measurable $InsP_3$ was small and transient even in cells differentiated with dbcAMP, total InsP accumulation in the presence of lithium chloride (10mM) [27, 28] was measured to ensure that any transient generation of InsP was not missed by sampling at specified time points. The results are shown in Figure 1b. In dbcAMP differentiated cells, aggregation of Fcγ receptors initiated an increase in InsPs that was detectable at the first time point tested (5 min), and steadily increased in a linear fashion thereafter.

Surprisingly, InsPs were not increased above control values in cells treated with IFN-γ even 20 mins after receptor aggregation (Figure 1b).

These data indicate that the ability of Fcγ receptors to couple to InsP generation following receptor aggregation in these cells depends on the differentiation state of the cell. In U937 cells differentiated with dbcAMP, Fcγ receptors are coupled to $InsP_3$ generation whereas, in IFN-γ treated cells, they are not. Indeed, the failure to detect any InsPs accumulation in IFN-γ treated cells suggests that the calcium spike previously documented in these cells is induced in an $InsP_3$-independent manner.

Figure 2.1 Differential generation of inositol phosphates following ligand activation of Fcγ receptor in dbcAMP and IFN-γ treated cells.



a. InsP3 levels in dbcAMP or IFN-γ treated cells at set times (30 sec to 20 min) after Fcγ receptor aggregation. In dbcAMP-differentiated cells, InsP3 concentrations were above control (samples with no aded cross-linking antibody) values at all time points after Fcγ receptor aggregation, although concentrations appeared to oscillate. In IFN-γ-treated cells, InsP3 concentrations were never higher than control values at any time point. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of four different experiments.

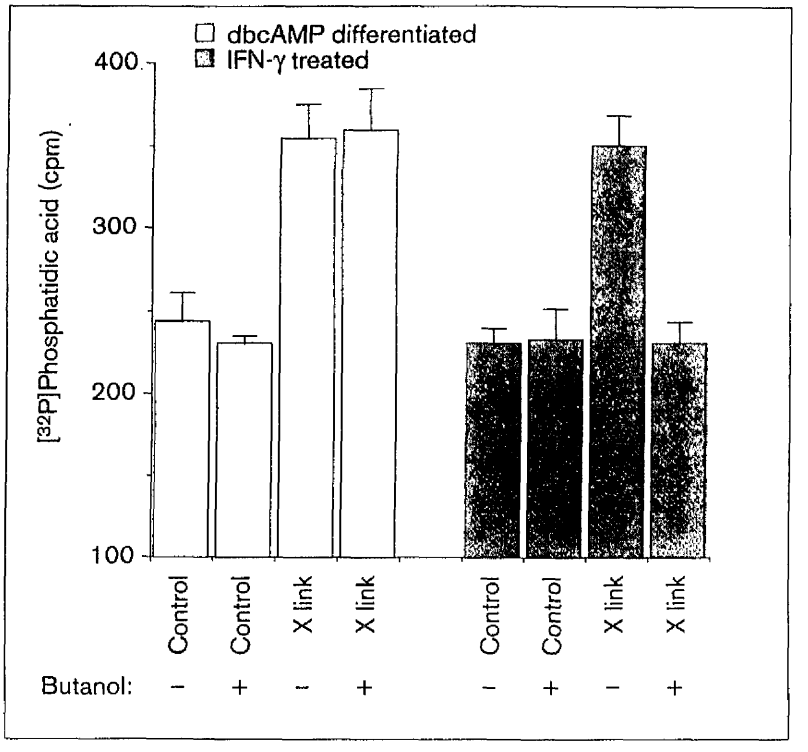
b. Total accumulated InsPs following Fcγ receptor aggregation in dbcAMP- and IFN-γ– treated cells. Cells were treated with 10mM lithium chloride to prevent breakdown of InsPs. Cells were harvested at 5 mins intervals after Fcγ receptor aggregation and assayed for InsPs concentration. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of five separate experiments.

2.4.1.2 *Fcγ receptor is coupled to DAG production in both differentiation states*

Our first clue to the potential calcium-mobilising signalling pathway utilised in IFN-γ treated cells was provided by experiments measuring mass diacylglycerol (DAG) generation. Unexpectedly, in contrast to the InsPs measurements, broadly similar levels of DAG were produced by Fcγ receptor aggregation in cells treated with IFN-γ or cells differentiated with dbcAMP (Figure 2). As DAG can be generated by activation of the alternative phospholipases PtdCho-PLC and PtdCho-PLD (from phosphatidic acid (PtdOH) via phosphatidic acid phosphohydrolase) [28] in addition to that resulting from $PtdInsP_2$-PLC hydrolysis, we next investigated whether either of these alternative pathways was responsible for the DAG generation. To do this, DAG was measured in cells pretreated with butan-1-ol (0.3%) which traps phosphatidic acid (PtdOH), the immediate product of PtdCho-PLD, in a non-hydrolysable form as phosphatidyl butanol (PtdBut) [28]. A control for the specificity of this reaction is provided by the stereoisoform, butan-2-ol which cannot act as an acceptor for the phosphatidyl-moiety of PtdCho. In cells treated with IFN-γ, Fcγ receptor-mediated DAG production was completely blocked by butan-1-ol whereas, in cells differentiated with dbcAMP, DAG production was completely unaffected (Figure 2). Butan-2-ol had no effect on DAG production in either cell type (data not shown).

These results suggest that in dbcAMP differentiated cells DAG was, as expected from the InsP results, likely to be predominantly derived from $PtdInsP_2$-PLC signalling. In contrast, in IFN-γ treated cells DAG generation was entirely derived from coupling of Fcγ receptors to a PLD pathway.

Figure 2.2 Fcγ receptor is coupled to diacylglycerol production in both differentiation states.



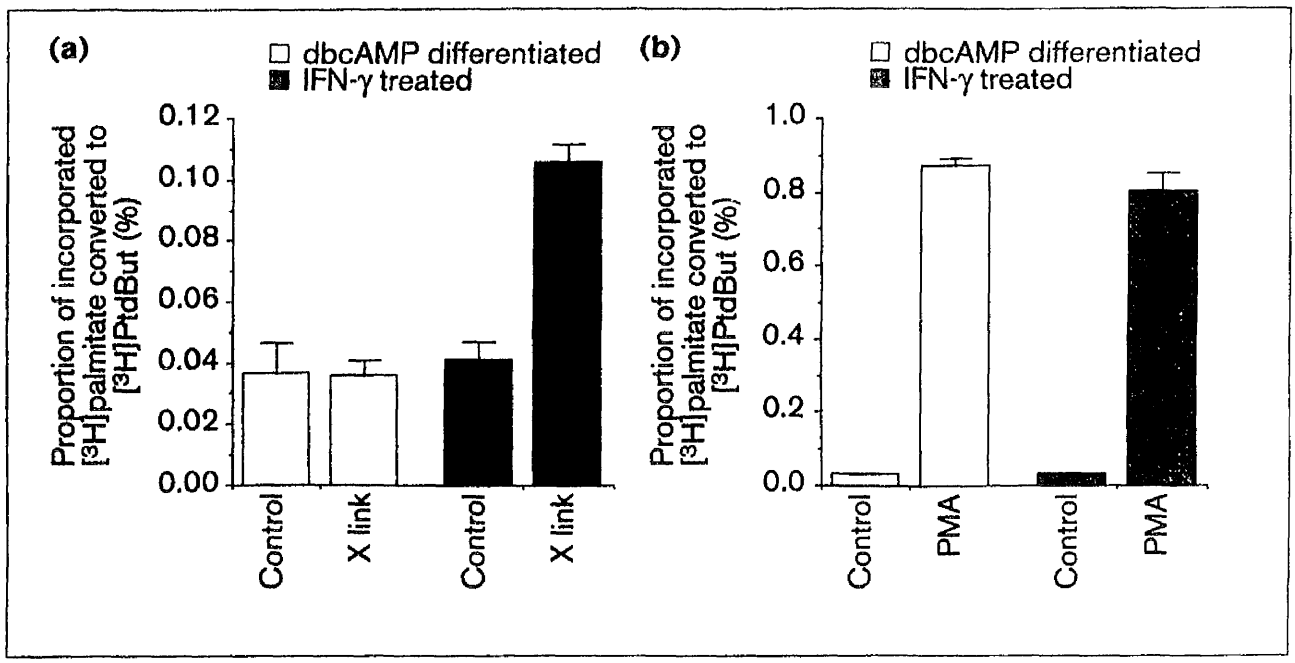
Relative DAG mass levels after aggregation of Fcγ receptor in dbcAMP-differentiated and IFN-γ-primed cells. The effect of butan-1-ol (0.3%) on the levels of DAG mass before (Control) and after (X link) Fcγ receptor aggregation was examined. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of four separate experiments.

2.4.1.3 *Fcγ receptors are coupled to PLD in IFN-γ treated cells but not in dbcAMP differentiated cells*

To confirm that Fcγ receptor signalling in IFN-γ treated cells is indeed coupled to a PtdCho-PLD pathway, we investigated coupling in [^{3}H]palmitate-labelled U937 cells using the transphosphatidylation assay [27]. These experiments definitively showed that aggregation of Fcγ receptors in IFN-γ treated cells stimulated activation of PtdCho-PLD, as evidenced by substantial generation of [^{3}H]PtdBut in butan-1-ol (Figure 3), but not butan-2-ol (data not shown) treated cells over 30 min. By contrast, no accumulation of [^{3}H]PtdBut was detected in cells differentiated with dbcAMP (Figure 3). To ensure that differentiation with dbcAMP had not down-regulated PLD activity in these cells, the level of activity following stimulation with the phorbol ester, PMA (phorbol 12,13 myristate acetate) was compared in differentiated cells and those treated with IFN-γ (Figure 3b). In both cell types, PMA (1μM) stimulated PLD essentially to an equivalent extent indicating that although Fcγ receptors are not coupled to PtdCho-PLD in dbcAMP-differentiated cells, one or more PtdCho-PLD activities are indeed expressed in these macrophage-like cells.

These results, therefore, categorically show differential coupling of the Fcγ receptors to major phospholipid signalling pathways, PtdInsP_2-PLC and PtdCho-PLD, at different stages of monocyte differentiation. Moreover, this switch in lipid signals is absolute.

Figure 2.3 Fcγ receptor is coupled to PLD in IFN-γ-treated cells but not in dbc-differentiated cells.



Measurement of PLD activity in dbcAMP and IFN-γ treated cells using the transphosphatidylation assay.

a. PLD activity was measured as the accumulation of [^{3}H]PtdBut in cells before (Control) and after (X link) aggregation of Fcγ receptors. The time for the accumulation assay was 30 min. Significant PLD activity was only observed in IFN-γ treated cells after Fcγ receptor aggregation

b. PLD activity in dbcAMP or IFN-γ-treated cells following activation by phorbol ester, (PMA).

The data shown are the mean $\pm$ the standard deviation of triplicate measurements and are representative of six separate experiments.

2.4.1.4 *FcγRI-mediated calcium transients in IFN-γ treated cells are dependent on phospholipase D activation and sphingosine kinase*

As no $InsP_3$ accumulation could be detected in IFN-γ treated cells following aggregation of FcγRI, alternative routes for calcium release were investigated. As a sphingoid base has previously been implicated in mobilisation of intracellular calcium stores [29-31], the differentiation-dependent coupling of FcγRI to sphingosine kinase was investigated in these cells. A prompt two fold increase in sphingosine kinase activity was observed 30 secs following receptor aggregation in these cells whereas it was not activated in dbcAMP differentiated cells.

A specific link between sphingosine kinase activation and PtdCho-PLD was demonstrated by pretreating cells with 0.3% butan-1-ol which completely abolished the increase in sphingosine kinase activity observed after specific aggregation of FcγRI in IFN-γ treated cells whereas butan-2-ol had no effect on sphingosine kinase activation (data not shown). This clearly demonstrates that PtdCho-PLD activation is upstream of sphingosine kinase; a finding that is consistent with the previous observation that phosphatidic acid, the immmediate product of PtdCho-PLD activation, can stimulate sphingosine kinase *in vitro* [32].

The role of components of the novel activation pathway (PtdCho-PLD and sphingosine kinase) identified in IFN-γ primed cells in mediating the calcium transients was determined. Consistent with a role for sphingosine kinase, treatment of these cells with D-L-*threo* dihydro-sphingosine (25 M) (DHS) completely abolished the release of calcium from stores initiated by aggregating FcγRI in IFN-γ but not dbcAMP-treated cells. Furthermore, preincubation of IFN-γ primed cells for 5 min with 0.3% butan-1-ol decreased the peak cytosolic calcium mobilisation from 217 $\pm$ 30 nM in the untreated cells to 99 $\pm$ 29 nM.

2.4.1.5 *Summary for section 1*

Taking all this data together, we have shown that immune complexes trigger different phospholipid signalling pathways dependent on the differentiation state of the cell. In dbcAMP differentiated cells, PtdInsP$_2$-PLC is activated and calcium mobilised through the conventional pathway involving InsP$_3$. However, in interferon-γ primed cells, a novel pathway has been identified that involves sequential activation of PtdCho-PLD and sphingosine kinase and mobilisation of calcium through an InsP$_3$ independent route. The immediate product of PtdCho-PLD, phosphatidic acid, has previously been shown to be an activator of sphingosine kinase in other systems [32] suggesting that phospholipase D may be directly upstream of sphingosine kinase. Moreover, the calcium transients were inhibited by both DHS and butanol indicating that this pathway generates second messengers able to release calcium from stores through a novel mechanism presumably involving a sphingoid base acting on the recently cloned SCaMPER receptor [33] in the endoplasmic reticulum to effect calcium release from stores [29]. This novel pathway may, therefore, play an important role in the regulation of calcium in a wide range of biological responses.

2.4.2 Section 2: mechanism of the switch

2.4.2.1 *The switch in phospholipid signalling pathways observed after differentiation is a function of the Fcγ receptor aggregated*

The switch in the phospholipid signalling pathway recruited by immune complexes may result from the influence of differentiation on any one of the components of the pathway. To investigate whether the switch in phospholipase-activated signalling is defined by the nature of the predominant Fcγ receptor on the cell surface, monoclonal antibodies specific

for either FcγRI or FcγRIIa were used to cross–link each receptor (see section **2.2.2** for more details). We compared the results of cross–linking the individual receptors with those obtained by immune–complex activation in both IFN–γ and dbcAMP–differentiated cells.

In dbcAMP differentiated cells, specific aggregation of either FcγRI or FcγRIIa gave similar results to those observed using human IgG, namely a measurable rise of InsPs (Figure 4a,b) and DAG (Figure 4c,d); the accumulation of the latter was unaffected by pretreating the cells with 0.3% butan-1-ol. In keeping with these results, no increase in PLD activity was measured after cross-linking either receptor in these differentiated cells (Figures 4e). To rule out the possibility that FcγRI signalling here simply reflected recruitment of FcγRIIa by the Fc region of either anti-FcγRI monoclonal antibodies used (antibodies 22 and 32), which are both mouse IgG1 subclass and can can be recognised at low affinity by FcγRIIa [34], the level of inositol phosphates was measured in dbcAMP-differentiated cells in the presense of either whole molecule intact monoclonal antibodies 22 and 32 or a $F(ab')_2$ preparation of the same antibodies. Polyclonal mouse IgG was used as an additional control. The intact anti-FcγRI antibodies and their $F(ab')_2$ fragments gave an identical accumulation of inositol phosphates whereas mouse IgG1 did not stimulate the generation of inositol phosphates (Figure 4f). Thus, in dbcAMP differentiated cells, both FcγRI and FcγRIIa appear to be coupled to PtdInsP_2-PLC and neither receptor is coupled to PtdCho-PLD. These results agree with those seen when Fcγ receptors are aggregated by immune complexes in dbcAMP-treated cells.

Legend for figure 2.4

Response of dbcAMP differentiated cells to specific crosslinking of FcγRI or FcγRIIa using monoclonal antibodies.

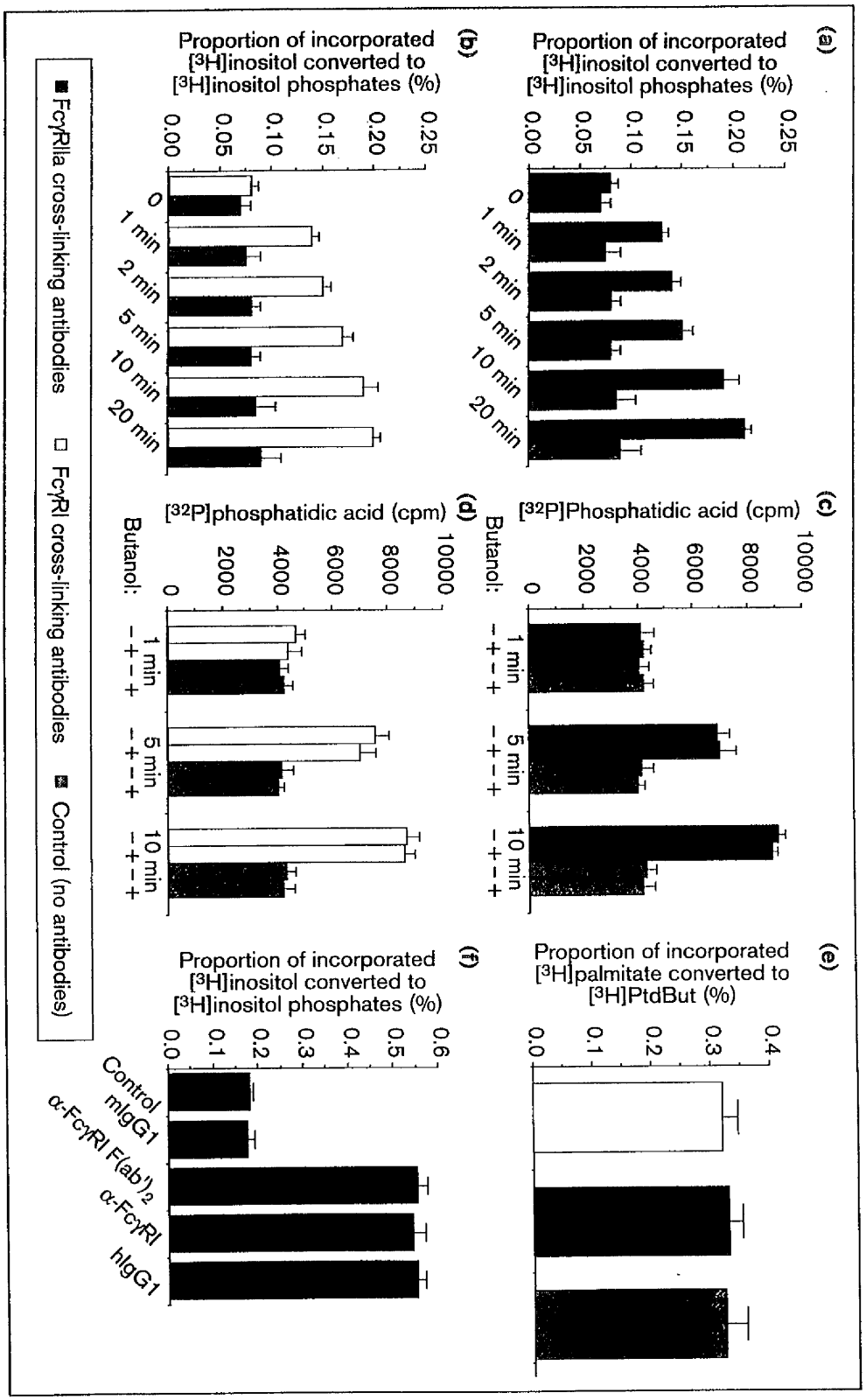
a, b. Accumulated inositol phosphates at specified times after specific receptor cross-linking. The results are the mean ± the standard deviation of triplicate measurements are representative of four separate experiments.

c, d. DAG generation after specific receptor cross-linking for the indicated times and the effect of (Butanol) butan-1-ol on DAG production. The results are the mean ± the standard deviation of triplicate measurements are representative of four separate experiments.

e. PLD activity after specific receptor crosslinking. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of five separate experiments.

f. Comparison of the ability of intact anti-FcγRI monoclonal antibodies and $F(ab')_2$ preparations to induce inositol phosphate generation in dbcAMP-differentiated cells. In the presence of 10mM LiCl, cells were loaded with either no primary antibody (Control), or equivalent concentrations of polyclonal monomeric mouse IgG1 (mIgG1), the $F(ab')_2$ preparations of the FcγRI-specific monoclonal antibodies 22 and 32 (α-FcγRI ($F(ab')_2$), intact whole molecule 22 and 32 (α-FcRI) or polyclonal monomeric human IgG1 (hIgG1). Following addition of the appropriate secondary antibodies and warming of cells to 37°C for 20 mins, total inositol phosphates were measured. The results are the mean ± the standard deviation of triplicate measurements are representative of three separate experiments.

Figure 2.4 Response of dbcAMP differentiated cells to specific crosslinking of FcγRI or FcγRIIa using monoclonal antibodies.



In contrast, in IFN-γ treated cells, completely different patterns of signalling were obtained by cross-linking of specific Fcγ receptors. Only specific aggregation of FcγRI resulted in similar results to those for ligand activation, namely no detectable accumulation of inositol phosphates (Figure 5a,b), a rise in DAG which was abolished by pretreating cells with 0.3% butan-1-ol (Figure 5c,d) and an increase in PLD activity (Figure 5e). By contrast, specific aggregation of FcγRIIa in IFN-γ-treated cells using monoclonal antibodies gave an identical pattern of responses to that observed for dbcAMP differentiated cells, namely an increase in inositol phosphates (Figure 5a,b) and butan-1-ol-insensitive DAG generation (Figure 5c,d) with no increase in PLD activity (Figure 5e). Thus, in IFN-γ treated cells, FcγRI is coupled to PtdCho-PLD and FcγRIIa to $PtdInsP_2$-PLC.

Taken together, these data indicate that FcγRIIa always signals through the activation of $PtdInsP_2$-PLC regardless of the differentiation state of the cell. In contrast, FcγRI appears to switch its coupling to different phospholipid signalling pathways in a differentiation manner. In dbcAMP-differentiated-cells, FcγRI is coupled to $PtdInsP_2$-PLC and thereby adopts a signalling pattern that is identical to that observed for FcγRIIa. In IFN-γ treated cells, FcγRI is independently coupled to PtdCho-PLD and unlike FcγRIIa is not coupled to $PtdInsP_2$-PLC. This switch in coupling to phospholipid signalling pathways by FcγRI is absolute and is identical to that observed for ligand (human IgG) activation (Figures 1-3).

Legend for figure 2.5

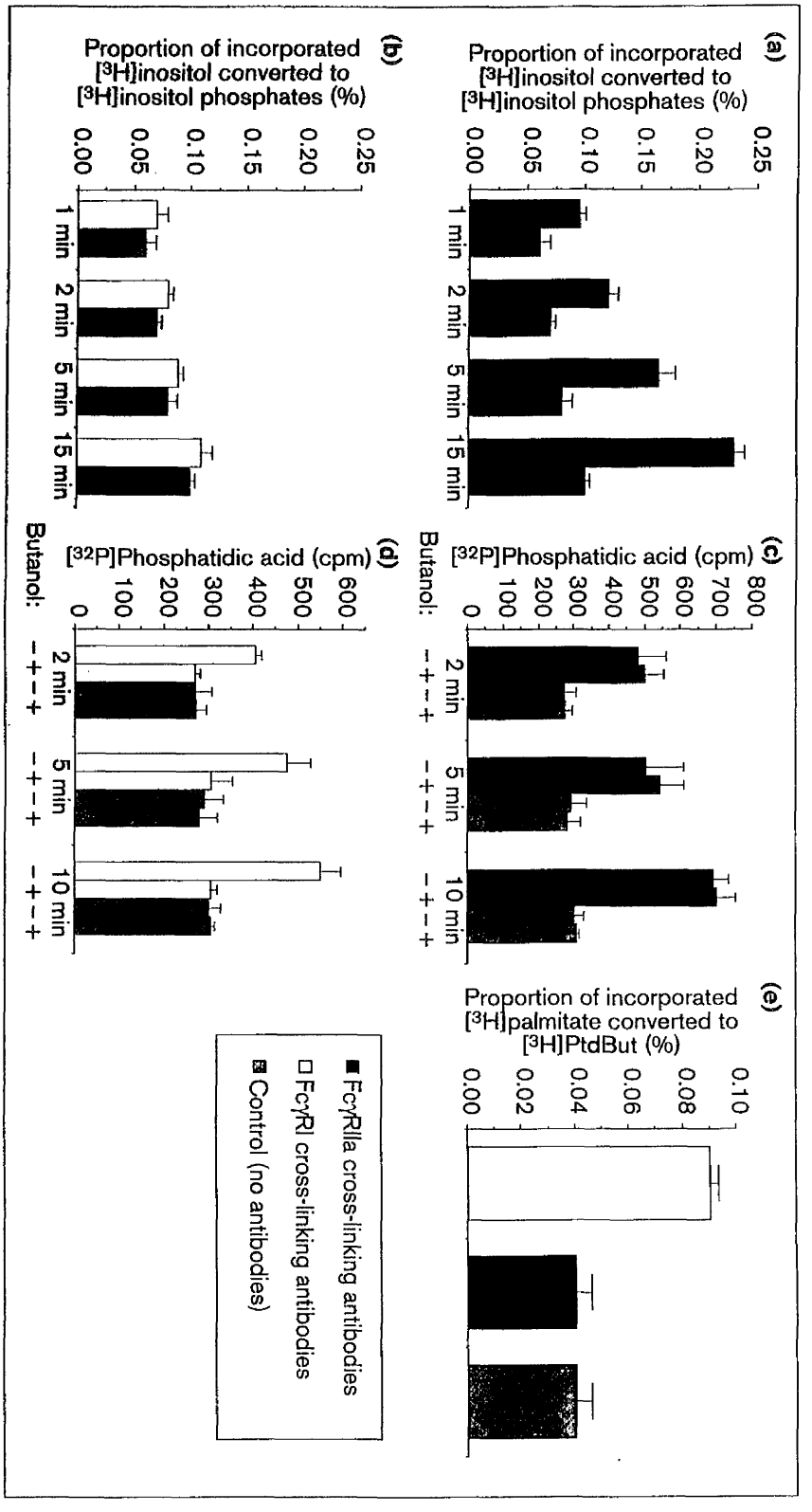
Response of IFN-γ-primed cells to specific crosslinking of FcγRI or FcγRIIa using monoclonal antibodies.

a,b. Accumulation of inositol phosphates at specified times after specific receptor cross-linking. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of six separate experiments.

c,d. DAG generation after specific receptor cross-linking for the indicated times and the effect of butan-1-ol (Butanol) on DAG production. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of four separate experiments.

e. PLD activity after specific receptor cross-linking. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of four separate experiments.

Figure 2.5 Response of IFN-γ-primed cells to specific crosslinking of FcγRI or FcγRIIa using monoclonal antibodies.

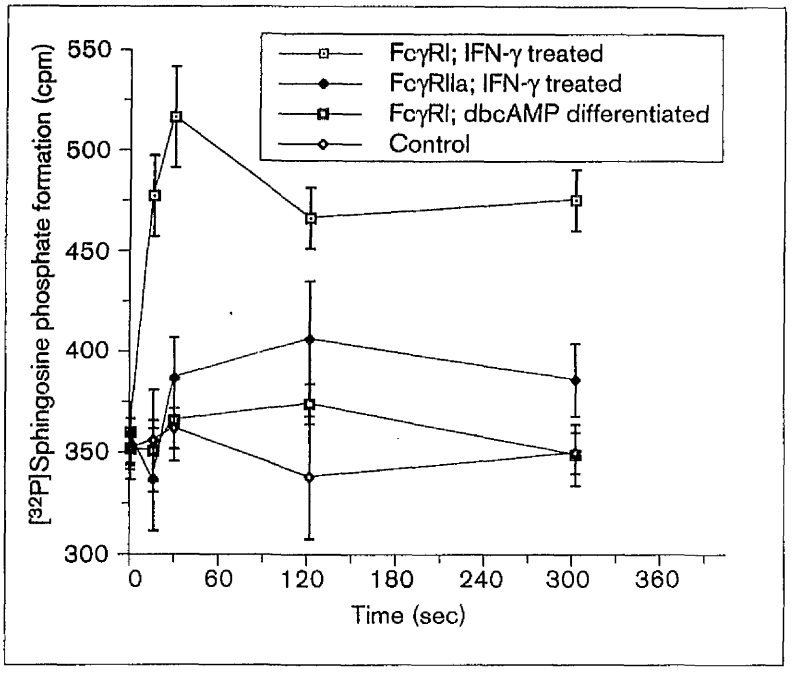


2.4.2.2 The *FcγRI-dependent pathway in IFN-γ treated cells is coupled to sphingosine kinase activation*

As our studies had linked the activation of PtdCho-PLD with sphingosine kinase, the coupling of specific Fcγ receptors to sphingosine kinase activation and the effect of differentiation on its activation were examined using the monoclonal antibodies to aggregation each receptorin specifically. Consistent with the identified switch in phospholipid signalling pathways, sphingosine kinase activity was stimulated only following the specific aggregation of FcγRI in IFN-γ primed cells (Figure 6). The sphingosine kinase activity did not rise above control values following aggregation of FcγRIIa in IFN-γ -treated cells or following aggregation of either FcγRI or FcγRIIa in dbcAMP-differentiated cells (Figure 6). Thus, the receptor specificity of sphingosine kinase activation exactly parallels that observed for activation of PtdCho-PLD (Figure 4e and 5e).

This data therefore indicates that, in IFN-γ-treated cells, FcγRI is coupled to a PtdCho-PLD and sphingosine kinase pathway whereas, in dbcAMP-treated cells, FcγRI is coupled to PtdInsP_2-PLC and production of InsP_3. In contrast, FcγRIIa is coupled to PtdInsP_2-PLC and InsP_3 in both cell types.

Figure 2.6 Sphingosine kinase activity is involved in the sinalling pathway triggered by FcγRI in IFN-γ primed cells.



Sphingosine kinase activity in cells treated with IFN-γ or dbcAMP followed by specific receptor (FcγRI or FcγRIIa) cross-linking using monoclonal antibodies.

Controls are IFN-γ-primed cells with no cross-linking antibody added. The data shown are the mean ± the standard deviation of triplicate measurements and are representative of three separate experiments.

2.4.2.3 *The switch in phospholipid signalling pathway is matched by a change in the nature of the calcium response*

Differentiation of U937 cells with dbcAMP changes the nature of calcium transient responses to Fcγ receptor aggregation stimulsted by immune complexes. Thus, in dbcAMP-differentiated cells, about 50% of cells respond in the form of calcium oscillations [26] whereas in undifferentiated or IFN-γ treated cells, the calcium response is in the form of a single spike. To determine whether the differential calcium responses observed following maturation reflect the change in the phospholipid signalling pathways recruited, we compared the nature of calcium response resulting from aggregation of either FcγRI or FcγRIIa using monoclonal antibodies with the response triggered by cross-linking with immune complexes. In dbcAMP differentiated cells, about 50% of cells respond in the form of calcium oscillations regardless of the nature of the Fcγ receptor aggregated (Figure 7a,panels c-g,). In cells treated with IFN–γ, the nature of aggregated receptor switched the nature of the calcium response (Figure 7d-g); thus, specific aggregation of FcγRI resulted in a single spike of calcium (Figure 7e), identical to that observed in response to human IgG (Figure 7d), and pecific cross-linking of FcγRIIa gave calcium oscillations in 50% of the cells, identical to those observed in dbcAMP differentiated cells (Figure 7a-c,f,g). Thus, the failure to observe calcium oscillations following ligand activation in IFN-γ treated cells is not a feature of their lack of the appropriate cellular machinery as, given the relevant trigger (FcγRIIa cross-linking), these cells are able to establish calcium oscillations. These data indicate that aggregation of FcγRIIa initiates calcium oscillations in U937 cells regardless of their differentiation status (Figures 7c,f,g). The finding that specific aggregation of FcγRI in the two differentiation states gives different calcium responses completely matches the switch observed differentiation-dependent switch in lipid signalling

pathways. Thus, the differentiation-dependent switch in the activated phospholipid pathway dictates the nature of the calcium transients.

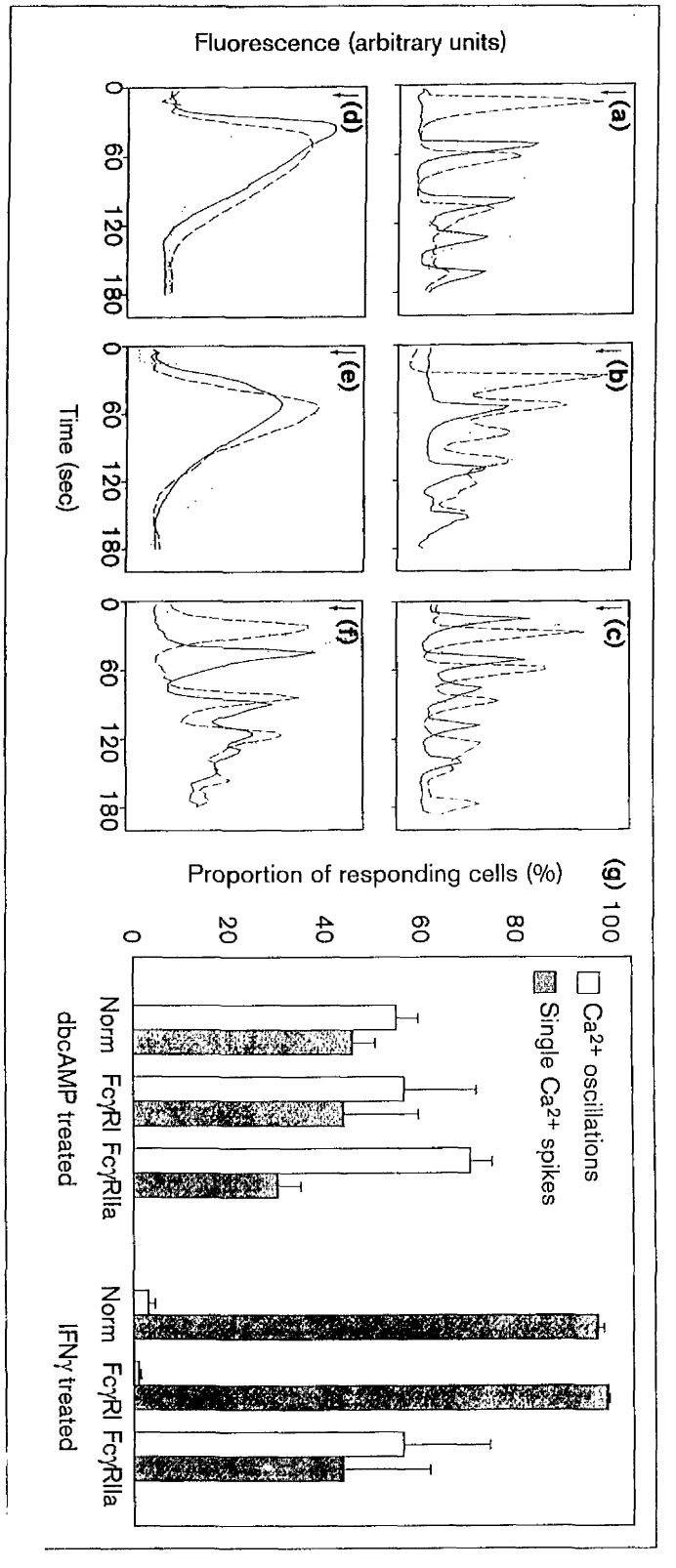
Legend for figure 2.7

Calcium responses in IFN-γ and dbcAMP treated cells following Fcγ receptor aggregation by ligand or cross-linking of specific Fcγ receptors using monoclonal antibodies.

a-f. Changes in Fluo3 fluorescence as an indication of cytosolic calcium - representative traces from three cells (from different experiments) in either (**a-c**) dbcAMP-differentiated cells or (**d-f**) IFN-γ –primed cells. (**a, d**) Fcγ receptor aggregation triggered by cross-linking monomeric human IgG; (**b, e**) cross-linking of FcγRI using specific monoclonal antibodies 22 and 32; (**c, f**) cross-linking of FcγRIIa using specific monoclonal antibody 2e1 on cells preloaded with human IgG4 to block the binding site of FcγRI (detailed in Materials and methods).

g. Statistical analysis of the proportion of cells responding in with calcium oscillations or a single spike of calcium under the conditions shown in (**a-f**). Each condition – cross-linking using IgG (norm), anti-FcγRI antibodies (FcγRI) or anti-FcγRIIa antibodies (FcγRIIa) was examined in four separate experiments. The mean value and standard error is given for each condition - at least 200 individual cells were analysed.

Figure 2.7 The switch in phospholipid signalling pathway is matched by a change in the nature of the calcium response.



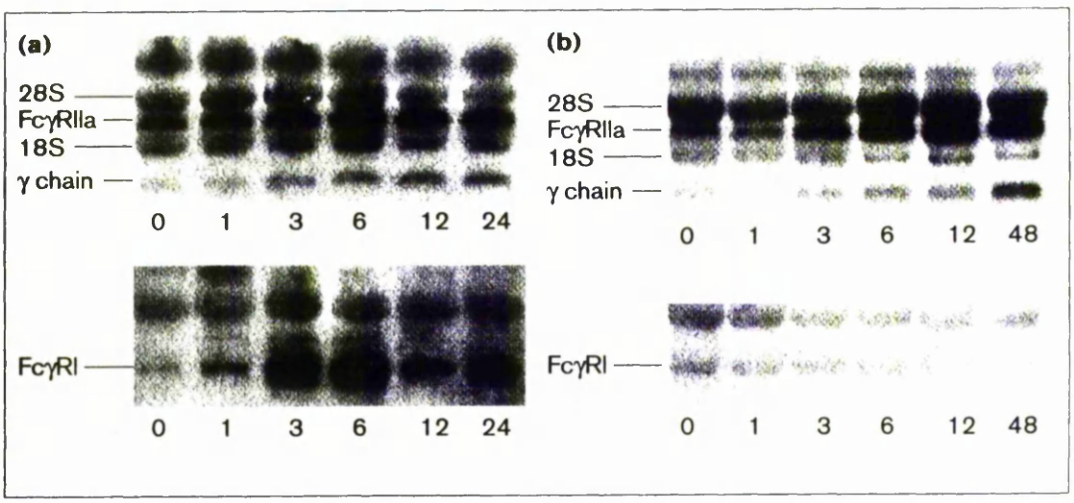
2.4.2.4 *Changes in FcγRIIa and γ chain expression upon IFN-γ and dbcAMP treatment are identical*

Activation of both phospholipid pathways depended on the initial activation of tyrosine kinases: pretreatment of cells with the tyrosine kinase inhibitor genistein (0.37 mM) completely abolished the rise in PtdCho-PLD and sphingosine kinase following FcγRI aggregation in IFN-γ treated cells (data not shown). To activate tyrosine kinases, FcγRI must recruit an accessory molecule as this receptor contains no ITAM in its cytoplasmic tail. We therefore investigated whether IFN-γ and dbcAMP differentially induced the γ chain and FcγRIIa, the two ITAM bearing molecules able to interact with FcγRI. Consistent with previous reports [7], IFN-γ resulted in a transient fifteen fold increase of FcγRI mRNA levels over basal levels (Figure 8a). This transient rise in mRNA markedly precedes the increase in surface expression of FcγRI [7]. IFN-γ also increases both surface FcγRIIa [12] and γ chain protein [14] in U937 cells. Consistent with these previous observations for protein expression, IFN-γ resulted in a transient increase of mRNA levels of both FcγRIIa and γ chain (Figure 8a). However, both these rises were much smaller (two-fold over basal levels) than that observed for FcγRI mRNA, although the time course of the response for all three transcripts was identical.

Treatment of cells with dbcAMP resulted in a steady decrease in the amount of FcγRI mRNA such that after 48 hours levels had fallen to 50% starting levels (Figure 8b); however, as the protein on the cell surface appears to be stable, surface FcγRI remains readily detectable on these cells [12]. In contrast, levels of FcγRIIa mRNA rose steadily over time. These results of differential control of FcγRI and FcγRIIa expression are consistent with the known relative pattern of surface expression of the two receptors [12]. Surprisingly, γ chain mRNA levels also rose during treatment of the cells with dbcAMP in an identical manner to that observed for FcγRIIa (Figure 8b). Thus, the

mRNAs encoding both ITAM-containing molecules behave in an identical fashion following cytokine treatment and differentiation. Regulation of the molecular switch in FcγRI signal transduction must be more complex than a mere change in the relative stoichiometry of FcγRI to either of its accesory molecules.

Figure 2.8 Changes in FcγRIIa and γ chain expression upon IFN-γ and dbcAMP treatment are identical.



Levels of mRNA for FcγRI, FcγRIIa and the γ chain.

a. U937 cells were treated with IFN-γ and cells harvested at 0, 1, 3, 6, 12 and 24 hours. Equal amounts of total RNA from each time point were electrophoresed through a formaldehyde 1% agarose gel. After transfer on to Nylon membranes, specific transcripts for FcγRI, FcγRIIa and γ chain were visualised usingthe relevant ^{32}P-labelled probes.

b. U 937 cells were treated with dbcAMP and cells harvested at 0, 1, 3, 6, 12 and 48 hours. Total RNA extracted from these cells was handled as in (**a**). The positions of 28S and 18S RNAs are indicated.

2.4.2.5 *Mechanism of the molecular switch in FcγRI mediated signalling following differentiation*

The role of the two accessory molecules (FcγRIIa and the γ chain) in signal transduction by FcγRI was next investigated by blocking the induction of expression using antisense oligonucleotides. In IFN-γ treated cells, loading cells with antisense γ chain completely abolished the increase in PtdCho-PLD activity observed following specific aggregation of FcγRI with monoclonal antibodies (Figure 9a). Loading cells with an equivalent antisense oligonucleotide to FcγRIIa had no influence at all on FcγRI-stimulated activation of PtdCho-PLD (Figure 9a). In IFN-γ treated cells, we have shown that specific aggregation of FcγRIIa activates $PtdInsP_2$-PLC with generation of InsP (Figure 5a). Therefore, to check the specificity of these antisense oligonucleotides, their ability to interfere with FcγRIIa-mediated activation of $PtdInsP_2$-PLC was investigated. The normal increase in inositol phosphstes observed after specific aggregation of FcγRIIa was blocked in cells loaded with the oligonucleotide antisense to FcγRIIa (Figure 9b). The oligonucleotide antisense to the γ chain had no influence on the ability of FcγRIIa to activate $PtdInsP_2$-PLC (Figure 9b). These data therefore, definitively demonstrate that FcγRI is coupled to PtdCho-PLD activation through the γ chain in IFN-γ -treated cells and that FcγRIIa plays no role in signal transduction for the high affinity receptor in these cytokine primed cells. In cells differentiated to macrophage type using dbcAMP, FcγRI switches the nature of the activated intracellular signalling patway by switching the accessory molecule used for signal transduction. Thus, loading differentiated cells with antisense FcγRIIa completely abolished the accumulation of InsPs observed after aggregation of either FcγRIIa or FcγRI (Figure 9c). In contrast to the results in IFN-γ treated cells, loading these differentiated cells with antisense γ chain had no effect on FcγRI-mediated signalling (Figure 9c). These data therefore, definitively

demonstrate that following differentiation FcγRI recruits FcγRIIa for signal transduction.

The antisense oligonucleotides act as reciprocal internal controls for each other as neither antisense oligonucleotide influenced the receptor activation of the alternative pathway (Figure 9a-c). Moreover, surface expression of total immunoreactive FcγRII was reduced by some 30% in cells treated with the oligonucleotide antisense to FcγRIIa (data not shown). As monoclonal antibodies to FcγRII are unable to distinquish between the six potential isoforms of FcγRII, this observation likely represents a considerable underestimate of the specific effect of the antisense oligonucleotide on FcγRIIa, the only ITAM bearing form of FcγRII. Finally, for both pathways, a standard "jumbled" antisense control oligonucleotide was also assessed for nonspecific effects. Loading cells with this oligonucleotide did not influence the activation of either pathway (Figures 9d,e). Taken together, these data clearly demonstrate that the differentiation-dependent switch in signalling pathways activated by aggregated FcγRI results from a change in its recruitment of accessory molecules.

Legend for figure 2.9

Loading of cells with antisense oligonucleotides demonstrates that FcγRI is coupled to PtdCho-PLD through the recruitment of the γ chain in IFN-γ treated cells but is coupled to $PtdInsP_2$-PLC through FcγRIIa in dbcAMP differentiated cells.

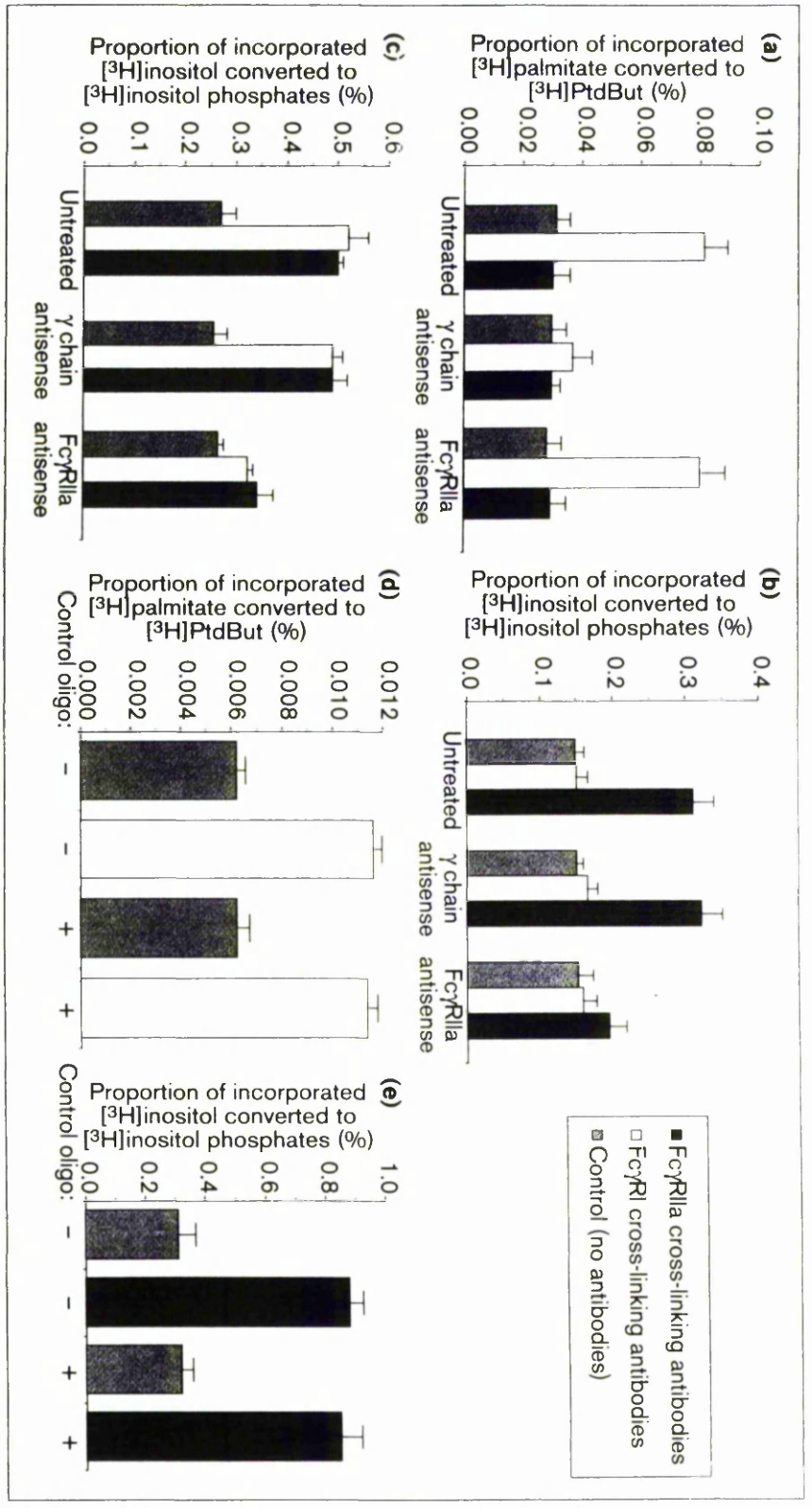
a,b U937 cells were loaded either with antisense γ chain or antisense FcγRIIa prior to treatment with IFN-γ overnight. Specific aggregation of FcγRI or FcγRIIa was achieved using monoclonal antibodies (see Materials and methods for more details). PtdCho-PLD activity (**a**) and the accumulation of inositol phosphates 20 min after receptor aggregation (**b**) were measured.. The data shown are the mean ± standard deviation of triplicate measurements.

c. U937 cells were loaded with antisense γ chain or antisense FcγRIIa prior to treatment for for 48 hours with dbcAMP (1 mM). The total accumulation of inositol phosphates was measured in cells 20 min after the specific aggregation of FcγRI or FcγRIIa using monoclonal antibodies. The data shown are the mean ± the standard deviation of triplicate measurements.

d. IFN-γ primed U937 cells were loaded with a jumbled antisense control oligonucleotide and the effect on FcγRI coupling to PtdCho-PLD was assessed.

e. dbcAMP-differentiated U937 cells were loaded with the jumbled antisense control oligonucleotide and the effect on FcγRIIa coupling to $PtdInsP_2$-PLC was assessed.

Figure 2.9 Mechanism of the molecular switch in FcγRI mediated signalling following differentiation.



2.4.2.6 *Summary for section 2*

The notable findings from this study are, firstly, that the two Fcγ receptors (FcγRI and FcγRIIa) initiate fundamentally different phospholipid signalling pathways in IFN-γ treated cells. Both pathways are present, intact and functional in IFN-γ treated cells but the two Fcγ receptors are differentially and exclusively coupled. The second finding is that the phospholipid pathway activated by specific cross-linking of FcγRI in monocytic and macrophage-like cells exactly mimics the differentiation-dependent switch in intracellular signalling observed for immune complex activation and results from a switch in the ability of this receptor to recruit different ITAM-bearing accessory molecules following differentiation.

2.5 CONCLUSIONS

We have shown that there is a fundamental and absolute switch in the nature of the intracellular signalling pathway activated by immune complexes when a monocytic cell line differentiates to a more macrophage-like cell. Thus, in cells primed with IFN-γ, the release of of intracellular calcium from stores is $InsP_3$ independent and is mediated by the sequential activation of PtdCho-PLD and sphingosine kinase. This newly identified signalling pathway presumably involves a sphingoid base acting on the recently cloned SCaMPER receptor [34] in the endoplasmic reticulum to effect the release of calcium from stores [29]. In dbcAMP-differentiated cells, the same immune complex challenge resulted in the activation of only $PtdInsP_2$-PLC; activation of the novel pathway involving PtdCho-PLD and sphingosine kinase could not be detected.

The ability of FcγRI to switch signalling pathways reflects the fact that, unlike FcγRIIa, the cytoplasmic tail of FcγRI contains no known signalling or ITAM motif but must recruit one of two accessory molecules. Therefore, unlike FcγRIIa, which through its intrinsic ITAM [33] demonstrates obligate coupling to PtdInsP$_2$-PLC, FcγRI is able to switch recruitment between the γ chain and FcγRIIa in a differentiation-dependent fashion and thereby couple to different intracellular signalling pathways. In dbcAMP-differentiated cells, FcγRI recruits FcγRIIa whereas, in cytokine primed cells, FcγRI uses the γ chain. The control of this switch does not rely on regulationof levels of γ chain but rather appears to depend on the relative levels of FcγRI and FcγRIIa. The activation of different signalling pathways probably accounts for the different responses of myeloid cells to challenge with immune complexes. As many immune receptors have separate polypeptide chains for ligand binding and signal transduction (allowing a similar switch in signalling pathways) the machanism defined here is likely to be widely used.

Implications for myeloid function of the differential signalling by Fc Receptors

The finding that differentiation to a more macrophage cell type causes U937 cells to switch the signalling pathways activated by Fcγ receptors such that prolonged calcium signals are generated agrees with our previous finding that dbcAMP upregulates capacitative calcium entry [35, 36], which is required for refilling of the calcium stores. It is tempting to speculate that the differentiation process primes cells to produce a prolonged response to immune complex activation by switching the nature of the second messenger to InsP$_3$ and inducing capacitative calcium entry to allow store filling, thereby maintaining the calcium signal. Another likely immediate consequence of this switch in phospholipase activation and calcium signalling is the

differential activation of protein kinase C isoforms, which are involved in a number of myeloid functions such as phagocytosis and endocytosis [37]. Such differential protein kinase C signalling may explain the finding that FcγRI-triggered phagocytosis can be inhibited by buffering cytoplasmic calcium [38]. Thus, this fundamental and absolute molecular switch in the activation of phospholipid signalling pathways following differentiation of cells to a macrophage cell type provides a method of precisely controlling the nature of the cellular response to immune complexes.

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CHAPTER 3

FcγRI-COUPLING TO PHOSPHOLIPASE D INITIATES SPHINGOSINE KINASE MEDIATED CALCIUM MOBILISATION AND VESICULAR TRAFFICKING

3.1 ABSTRACT

Aggregation of receptors specific for the constant region of immunoglobulin G activates a repertoire of monocyte responses that can lead ultimately to targeted cell killing via antibody directed cellular cytotoxicity. The high affinity receptor, FcγRI, contains no recognised signalling motif in its cytoplasmic tail but rather utilises the γ chain of $Fc_{\varepsilon}RI$ as an accessory molecule to recruit tyrosine kinases for signal transduction. We show here that, in a human monocytic cell line primed with interferon-γ , FcγR I mobilises intracellular calcium stores using a novel pathway that involves tyrosine kinase coupling to phospholipase D and resultant downstream activation of sphingosine kinase. Moreover, FcγRI is not coupled to phospholipase C and hence, calcium release from intracellular stores occurred in the absence of any measurable rise in inositol trisphosphate. Finally, as this novel activation pathway is also shown to be responsible for mediating the vesicular trafficking of internalised immune complexes for degradation, it is likely to play a key role in controlling intracellular events triggered by FcγRI.

3.2 INTRODUCTION

The macrophage-specific receptor (FcγRI) for the constant region (Fc) of immunoglobulin G (IgG) plays a central role in the clearance of immune complexes (1,2). FcγRI belongs to a family of receptors for IgG which are distinquished by the affinity for ligand. Whilst FcγRI is a high affinity IgG receptor, FcγRII and FcγRIII are both low affinity IgG receptors (reviewed in refs 1 and 2). Aggregation of FcγRI activates macrophages to undergo a repertoire of responses that can ultimately lead to cell killing through the process of antibody directed cellular cytotoxicity (ADCC); a critically important feature in the body's defence against virus infected cells and in cancer surveillance (3,4). Immune complex aggregation of FcγRI initiates signal transduction events which include protein tyrosine phosphorylation (5,6) and tyrosine kinase dependent calcium transients (7,8). However, the cDNA for FcγRI predicts an integral type I glycoprotein in which, unlike FcγRIIa, the cytoplasmic tail contains no recognised signalling motifs (9). FcγRI has been shown to associate non-covalently with the signal transducing γ chain (10) which contains an immunoreceptor tyrosine activation motif (ITAM) (11,12) in its cytoplasmic tail and this association is thought to allow aggregated FcγRI to recruit and activate soluble tyrosine kinases (13). The γ chain was originally identified in mast cells as a component of the high affinity IgE receptor, FcεRI, but has subsequently been found in macrophages in the absence of the α chain of FcεRI (14). Thus, although expressed in different cell types, the ligand recognition subunits (α chains) of FcγRI and FcεRI are able to use the same signal transducing molecule. Recently, FcεRI has been shown to mobilise calcium transients in a mast cell line through the activation of a novel pathway involving sphingosine kinase (15). However, the precise

details of the signalling pathway and its relationship to tyrosine kinase activation are as yet unclear.

In this study, we demonstrate that FcγRI mobilises calcium from intracellular stores by activating sphingosine kinase in the absence of phospholipase C activation and resultant generation of Inositol 1,4,5 triphosphate ($InsP_3$) . We also show that FcγRI-stimulated activation of sphingosine kinase is downstream of phospholipase D activation and both these enzymes are dependent on tyrosine kinase activation. Moreover, activation of this pathway is necessary and sufficient to account for intracellular calcium mobilisation after FcγRI aggregation in cytokine primed U937 cells and for efficient vesicular trafficking of internalised immune complexes for degradation.

3.3 MATERIALS AND METHODS

3.3.1 Receptor aggregation

U937 cells, a human monocyte cell line (16), treated with 200ng/ml interferon-γ for 18 hours were used for all experiments (8, 17). For the biochemical assays,approximately 3 x 10^6 cells were harvested and incubated with 1μM human monomeric IgG (Serotec) to occupy surface FcγRI. Unbound IgG was removed by dilution and centrifugation of the cells. The cells were resuspended in ice cold Hepes buffered saline (HBS) and cross-linking antibody (goat anti-human IgG 1:100 dilution) was added. The cells were then warmed to 37°C and harvested at specified times for biochemical assay. Where the low affinity receptor was specifically aggregated using anti-FcγRIIa, the cells were loaded with the monoclonal antibody 2e1 (1μg) (Serotec) in the presence of saturating concentrations (3μM) of human IgG4 (to block binding of the Fc portion of 2e1 to FcγRI). After removal of excess antibody, anti-FcγRIIa was aggregated by addition of goat anti-mouse IgG F(ab) (1:100 dilution).

3.3.2 Measurement of sphingosine kinase

Sphingosine kinase was assayed as described in reference 18. Briefly, reactions were terminated at the times specified in the figures by addition of ice-cold phosphate buffered saline (PBS). After centrifugation, the cells were resuspended in ice-cold 0.1M phosphate buffer (pH7.4) containing 20% glycerol, 1mM mercaptoethanol, 1 mM EDTA, phosphatase inhibitors (20mM $ZnCl_2$, 1mM sodium orthovanadate and 15mM sodium fluoride), protease inhibitors (10μg/ml leupeptin, 10μg/ml aprotinin and 1mM PMSF) and 0.5mM 4-deoxypyridoxine. Cells were disrupted by freeze thawing and

centrifuged at 105,000g for 90 min at 4°C. Supernatants were assayed for sphingosine kinase activity using sphingosine (Sigma) and [γ^{32}P]ATP (2μCi, 5mM) as specified in Olivera et al, 1994 (18). After incubation, products were separated by TLC on silica gel G60 using chloroform:methanol:acetic acid: water (90:90:15:6) and visualised by autoradiography. The radioactive spots corresponding to sphingosine phosphate were scraped and counted in a scintillation counter.

3.3.3 Measurement of sphingosine -1-phosphate

Sphingosine-1-phosphate concentrations were measured as described in reference 19. Briefly, cells were preincubated overnight (15h) in media containing [^{3}H]serine (20μCi/ml) to label cellular sphingolipids and free sphingosine pools. Following labelling, the cells were washed in ice cold RPMI 1640/10mM HEPES/0.1% BSA (RHB medium), and resuspended in ice cold RHB medium containing 0.1mM L-canaline and the pyridoxal phosphate analog 4-deoxypyridoxine (0.5mM) to inhibit the pyridoxal-dependent sphingosine-1-phosphate lyase. Cells were then stimulated by the addition of crosslinking antibody and warming to 37°C, the reactions terminated at specified times. Cells were harvested by centrifugation, the lipid were extracted and analysed by TLC on silica gel G60 using chloroform:methanol:acetic acid:water (90:90:15:6). Standard sphingosine-1-phosphate was applied with the samples and the lipids were visualised using iodine vapours. Bands corresponding to sphingosine-1-phosphate were excised from the plate and counted by liquid scintillation spectrometry. Results were calculated as per cent of the total radioactivity incorporated in the lipids. Data presented are the mean ± standard deviation of triplicate measurements and the results shown are representative of three different experiments.

3.3.4 Measurement of inositol phosphate

Inositol phosphates were assayed essentially as described in reference 20. Briefly, U937 cells were labelled with myo-[^{3}H]-inositol (1μCi/10^6 cells) for 16 hours at 37°C. The cells were washed three times and resuspended (at 1-3x10^7 cells/ml) in RPMI 1640/10mM HEPES/0.1% BSA (RHB) medium, pH7.4 at 4°C. Following stimulation, the cells were harvested, resuspended in 100μl HBS, transferred to glass trident vials and extracted by addition of 0.94ml chloroform:methanol (1:2) on ice for 10 min. A Bligh-Dyer phase separation was achieved by addition of 0.31ml chloroform and 0.31ml water, vortexing and centrifugation at 270g for 5 min. Levels of [^{3}H]inositol trisphosphate ($InsP_3$) or total [^{3}H]inositol phosphates (reaction mixture containing 10mM LiCl) were determined by liquid scintillation counting of fractions eluted following Dowex (formate form) ion-exchange chromatography of aliquots of the aqueous phase. Results were calculated as per cent of the total radioactivity incorporated in the lipids. Data presented are the mean ± standard deviation of triplicate measurements and the results shown are representative of three different experiments.

3.3.5 Measurement of DAG generation

Mass DAG was measured as described in reference 21. The lower organic phase of Bligh-Dyer extractions were dried *in vacuo* and the lipids solubilised in a Triton X-100/Phosphatidylserine mixture: Briefly, phosphatidylserine (30μl; supplied as 25mM stock from Lipid Products) is dried under nitrogen and then probe sonicated in 2.5ml of 10mM Imidazole buffer, pH6.6 containing 0.6% (w/v) Triton X-100, until the solution is optically clear. Aliquots (50μl) were added to the lipid samples which were then sonicated in a bath for 30 minutes. Once sonicated, 20μl of 250mM Imidazole buffer pH6.6 containing 250mM NaCl, 62.5mM $MgCl_2$, 5mM EGTA

and 10µl of freshly prepared 100mM DTT was added to the solubilised lipid. *E coli* diacylglycerol kinase (Calbiochem) was added to a final concentration of 50mUnit/ml and the reaction started by addition of 10µl of 5mM ATP containing 1µCi of [^{32}P]-γ-ATP made up in 100mM Imidazole pH6.6; this results in a final ATP concentration of 0.5mM in a final reaction volume of 100µl. The tubes are incubated at 30°C for 30 minutes. The reaction is stopped by addition of 1ml of chloroform:methanol: HCl (150:300:2). After 10 minutes, 300µl of chloroform and 400µl of H_2O are added. The tubes are vortexed and centrifuged at 270g for 5 min to promote phase splitting and washed once with 1ml of a synthetic upper phase. The samples were then dried *in vacuo*, solubilised in 40µl of chloroform:methanol (19:1) and 20µl spotted onto silica TLC plate (Merck, 5714, 5x20cm $60F_{254}$). The plates were developed in chloroform:methanol:acetic acid (38:9:4.5) and radiolabelled bands were located by autoradiography or phosphorimaging. The PtdOH band (relative to standards) was scraped into scintillation vials, scintillant added and the associated radioactivity determined by liquid scintillation counting.

3.3.6 Measurement of Phospholipase D Activity

PLD activity was measured by the transphosphatidylation assay (21). Briefly, U937 cells, were labelled (10^6 cells/ml) with [^{3}H]palmitic acid (5µCi/ml) in RPMI 1640 medium containing 5% (v/v) foetal calf serum for 16h. Following labelling, the cells were washed in ice cold RPMI 1640/10mM HEPES/0.1% BSA (RHB medium), resuspended at 2x10^6 cells/ml and incubated at 37°C for 15 mins in RHB medium containing butan-1-ol (0.3% final). Specific Fc receptors were crosslinked as described above and, after the times indicated, cells were extracted by Bligh-Dyer phase separation. The lower organic phase was removed, dried down under vacuum (Jouan RC1022), and the samples redissolved in 25µl chloroform: methanol (19:1 v/v), containing 40µg

unlabelled phosphatidylbutanol (Lipid Products, South Nutfield, Surrey, U.K.) as standard, and applied to pre-run, heat activated TLC plates (20 x 20 cm, Silica gel 150A grooved plates, Whatman). The plates were developed in the organic phase of the solvent, ethyl acetate: 2,2,4 trimethylpentane: acetic acid: water (11:5:2:10) for approximately 90 mins and the position of the phosphatidylbutanol product detected using iodine vapour. [^{3}H]PtdBut-containing silica indicated by the phosphatidylbutanol standard was then scraped into scintillation fluid and counted. Results were calculated as per cent of the total radioactivity incorporated in the lipids. Data presented are the mean $\pm$ standard deviation of triplicate measurements and the results shown are representative of three different experiments.

3.3.7 Measurement of tyrosine phosphorylation by Western blot

U937 cells were loaded with human IgG and cross-linked as described earlier. After washing in PBS the cells were lysed with ice-cold RIPA lysis buffer containing 1mM PMSF, 10µg/ml CLAP (10µg/ml each of chymostatin, leupeptin, antipain and pepstatin), 1mM sodium orthophosphate and 1mM sodium fluoride, for 30 min. Cellular debris was removed by centrifugation at 13,000 rpm for 15 min and the cell lysates were incubated with an agarose-conjugated anti-phosphotyrosine monoclonal antibody (clone 4G 10; Upstate Biotechnology) at 4°C overnight. Phosphotyrosine proteins were then harvested by centrifugation of the agarose beads and were then dissociated from the beads by boiling in sample buffer (22) containing 50 mM DTT for 15 min. Samples were run in a 10 % SDS-polyacrylamide gel (23). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2µM pore size) as described (24). The presence of tyrosine-phosphorylated proteins was then detected by Western blotting with a monclonal anti-

phosphotyrosine antibody (clone 4G10; Upstate Biotechnology). Westerns were developed using the ECL system (Amersham).

3.3.8 Measurement of cytosolic calcium

Cytosolic calcium was measured in cell populations at 37°C using a Cairn Research Spectrophotometer as described previously (8). Cells were loaded with Fura2 and human monomeric IgG in HEPES buffered saline (HBS) supplemented with 1mM Ca^{2+} to prevent depletion of calcium stores. After dilution and centrifugation to remove excess dye and antibody, the cells were resuspended in a small volume of HBS, 1mM Ca^{2+} to give a final density of 10^6 cells/100µl. From this, cells were added to stirred cuvettes containing 1.4ml of nominally Ca^{2+} free HBS (at 37°C) in a Cairn Spectrophotometer system (Cairn Research Ltd). Excitation wavelengths of 340, 360 and 380nm were provided by a filter wheel rotating at 35 Hz in the light path. Emitted light was filtered by a 485nm long pass filter and samples averaged to give a data point every 500ms. The backgound corrected 340/380 ratio was calibrated using the method of Grynkiewicz et al 1985 (25). Following each experiment, cells were lysed by the addition of 50µM digitonin in the presence of external 2mM Ca^{2+} to give an R_{max} value. R_{min} was subsequently determined by the addition of 20mM EGTA (pH7.4) in the presence of equimolar concentration of Tris base.

3.3.9 Measurement of endocytosis and rate of trafficking for degradation

IFN-γ treated cells were harvested and washed in phosphate buffered saline (PBS), 1% BSA. The cells were then loaded with ^{125}I-labelled IgG as described in reference 17. After removal of nonbound radiolabel by dilution and centrifugation, cross-linking antibody was added and the cells warmed to 37°C for given times.

Endocytosis: The rate of endocytosis was assessed by measuring the rate of internalisation of radiolabelled surface immune complexes. At time zero, triplicate aliquots of cells were harvested into ice cold PBS (pH7.4) and this was counted in a Packard gamma counter to provide a measure of the total counts bound to the cell surface. To measure the proportion of radiolabelled immune complexes internalised after incubation at 37°C, any surface bound radiolabelled immune complexes can be stripped from the cell by incubating the cells in ice-cold acidified PBS (pH2.0) (17). Radiolabelled immune complexes that have been internalised remain trapped inside the cell and cannot be released by this acid wash. Thus, to assess the rate of internalisation, aliquots of cells were transferred at given times into ice-cold acidified PBS (pH2.0) for 5 minutes to strip off cell surface radiolabelled immune complexes (17), the cells were then centrifuged and the pellets counted in a Packard gamma counter to yield the counts that had been internalised or cell associated counts. The cell associated counts for each time point were then expressed as the percentage of total counts bound at time zero to provide a measure of the rate of internalisation of the immune complexes.

Degradation: After warming the cells to 37°C for long time intervals, the proportion of cell associated counts was observed to fall. To determine whether this reduction in cell associated counts represented degradation of the immune complexes, the supernatant following the cell incubation was examined for the presence of trichloroacetic acid soluble radiolabel indicating that the radiolabelled IgG had been degraded. Thus, cells were also harvested at the same time points to measure the rate of degradation of the internalised counts. Cells were centrifuged, the supernatants harvested and trichloroacetic acid (TCA) was then added to these supernatants. After incubation on ice for 60 mins, the samples were centrifuged at 12,000g at 4°C and the supernatants were counted to provide a measure of the TCA soluble counts in the

supernatant. The results were expressed as percentage of the initial cell associated counts at time zero.

The results shown are the mean $\pm$ standard deviation of triplicate measurements and are representative of three different experiments.

3.4 RESULTS

3.4.1 Aggregation of FcγRI activates sphingosine kinase in a tyrosine kinase dependent manner

In interferon-γ (IFNγ) primed U937 cells, aggregation of FcγRI with surface bound immune complexes results in calcium transients in the form of a single spike (reference 8 and Figure 1A). The FcγRI-associated accessory transducing molecule, γ chain, has recently been reported to mobilise calcium via activation of sphingosine kinase when coupled to the high affinity IgE receptor, FcεRI (15). Thus, to compare the nature of this FcγRI-calcium response to that of FcεRI, the effect of D-L-*threo*-dihydrosphingosine (DHS) on the release of calcium from intracellular stores was determined. Pretreatment of cells with 25μM DHS completely abolished the FcγRI-mediated rise in cytosolic calcium indicating that intracellular calcium stores are mobilised in these cells in a similar fashion to that observed for FcεRI in mast cells (15). The calcium stores were intact in cells treated with DHS as subsequent addition of thapsigargin (250nM) resulted in a prompt increase in cytosolic calcium; thereby, demonstrating that the failure to observe a rise in calcium following aggregation of FcγRI in cells pre-treated with DHS was not secondary to depletion of intracellular calcium stores.

As DHS acts as a competitive inhibitor of sphingosine kinase, the activity of this enzyme after FcγRI aggregation was next assessed. Aggregation of FcγRI stimulated a prompt increase in sphingosine kinase activity which was detectable within 30secs (Figure 1B). Sphingosine kinase activation by FcγRI aggregation in these cytokine primed cells was dependent on tyrosine kinase activation as treatment of the cells with genistein (0.37mM) completely abolished the response (Figure 1B). Pretreatment with genistein at a lower concentration (0.1mM) also completely inhibited FcγRI activation of

sphingosine kinase (Figure 1C) although concentrations below this only resulted in partial inhibition (Figure 1C).

In parallel with the activation of sphingosine kinase, FcγRI aggregation resulted in a prompt increase in the concentration of sphingosine-1-phosphate in these cells (Figure 1D). The concentration of sphingosine-1-phosphate peaked 30 seconds after receptor aggregation and, although levels fell gradually thereafter, concentrations remained elevated above control values 5 mins after receptor aggregation. Pretreatment of cells with genistein (0.37mM) completely abolished the FcγRI-mediated increase in sphingosine-1-phosphate generation.

Legend for figure 3.1

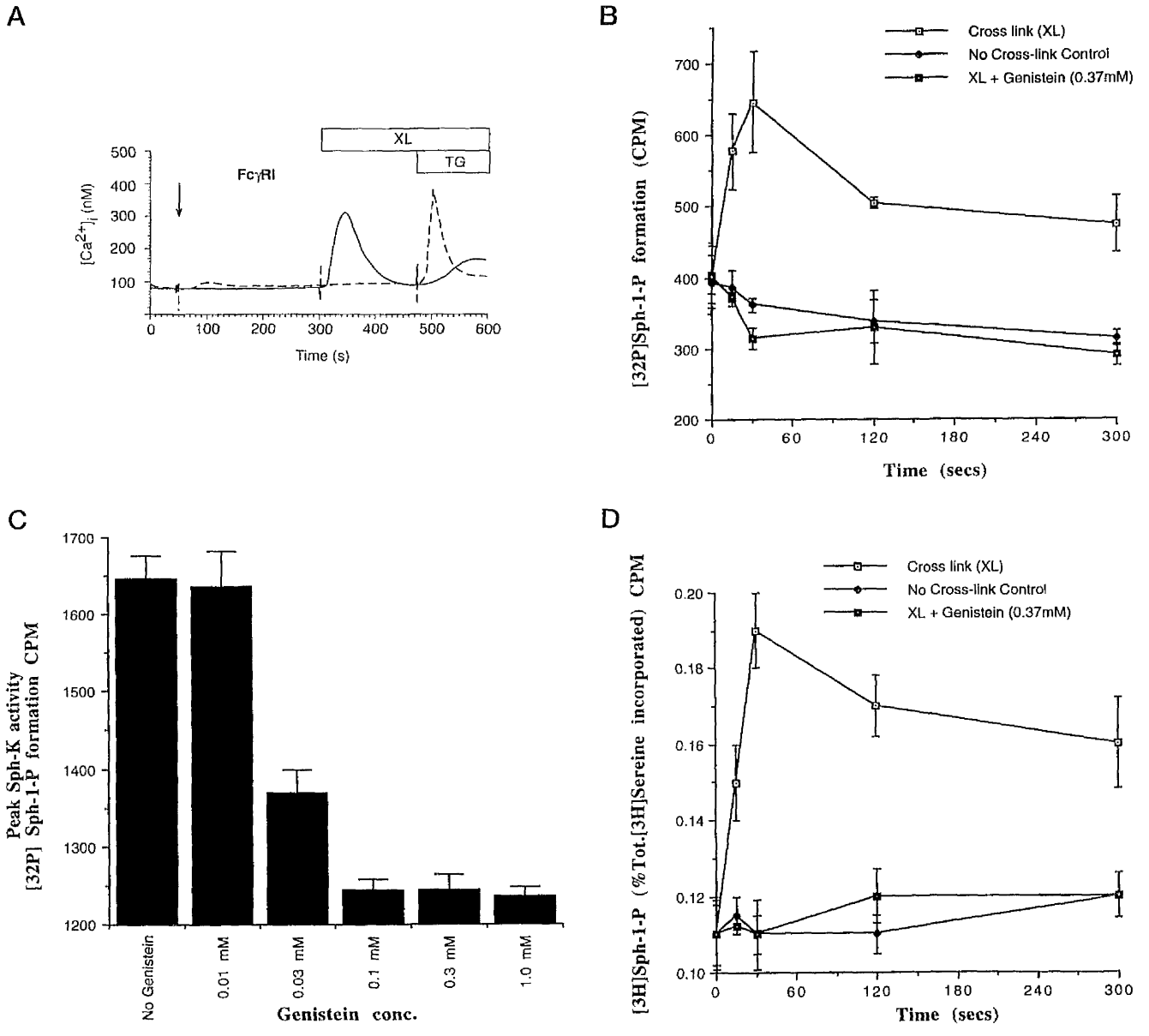
A. Cytosolic calcium concentrations in IFN-γ treated U937 cells following aggregation of FcγRI. The arrow indicates the addition of either 25μM DHS (dashed line) or vehicle (solid line). Crosslinking antibody (XL) was added to the cuvette at 300 secs and thapsigargin at 475 secs. Thapsigargin (250nM) was added to assess the viability of the stores. Typical trace from 5 separate experiments.

B. Activation of sphingosine kinase by FcγRI aggregation and the effect of tyrosine kinase inhibition. IgG-loaded FcγRI was aggregated by addition of crosslinking antibody (crosslink XL) and sphingosine kinase assayed in cell extracts at specified time points after aggregation. Results were compared to non-crosslinked controls (no crosslink control) and to cells pre-treated with genistein (0.37mM) for 30 mins prior to the addition of crosslinking antibody to inactivate tyrosine kinases (XL + genistein).
Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments

C. Effect of varying concentrations of genistein on sphingosine kinase activation by FcγRI aggregation. Sphingosine kinase activity was measured in cells 30 secs after aggregating FcγRI in cells pre-treated for 30 mins with varying concentrations of genistein (0.01, 0.03, 0.1, 0.3, 1.0 mM) and compared to untreated control cells.
Results shown are the mean ± S.D. for triplicate measurements at each concentration. The results shown are typical from three separate experiments

D. Increase in sphingosine-1-phosphate concentrations following FcγRI aggregation and effect of tyrosine kinse inhibition. Sphingosine-1-phosphate concentrations were measured in cells following aggregation of FcγRI (crosslink XL) and compared to non-crosslinked control cells (no crosslink control) and to cells pretreated with genistein (0.37mM) for 30 mins prior to addition of crosslinking antibody (XL + genistein).
Results shown are the mean ± S.D. for triplicate measurements at each time point.The results shown are typical from three separate experiments

Figure 3.1 Mobilisation of intracellular calcium stores by FcγRI associated with activation of sphingosine kinase and appearance of sphingosine-1-phosphate.



3.4.2 Aggregation of FcγRI activates phospholipase D and not phospholipase C in a tyrosine kinase dependent manner

As immune complex aggregation of FcγRI has previously been reported to lead to tyrosine phosphorylation of phospholipase Cγ1 (5) with presumed generation of $InsP_3$ and diacylglycerol (DAG), the role of this phospholipid signalling pathway in mediating the cytosolic calcium response was also investigated. Surprisingly, no increase in $InsP_3$ could be detected (data not shown). As $InsP_3$ generation can be transient in nature, the accumulation of total inositol phosphates (InsPs) was measured to ensure that any small transient InsPs signals did not go undetected. No accumulation of total InsPs over 20 minutes could be detected in IFN-γ primed U937 cells after aggregation of FcγRI (Figure 2A). PLC signalling was, however, functional in these cells as aggregation of a related immune receptor, the low affinity IgG receptor (FcγRIIa), using monoclonal antibodies resulted in a easily measurable accumulation of $InsP_3$ (data not shown) and total InsPs (Figure 2A). Unlike FcγRI, the low affinity receptor possesses an integral, albeit unconventional ITAM in its cytoplasmic tail: the tyrosine residues are separated by an unusually long intervening sequence (26). Taken together this data indicates that the high affinity receptor, FcγRI, mobilises calcium stores through a novel pathway that, unlike the low affinity receptor (FcγRIIa), does not involve $InsP_3$.

Interestingly, despite the lack of generation of InsPs over 20 mins, mass DAG concentrations were elevated following aggregation of FcγRI (Figure 2B). Thus, in an attempt to delineate alternative lipid signalling pathways involved in mediating the response to FcγRI, diacylglycerol (DAG) was measured in the presence of 0.3% butan-1-ol to block the generation of DAG derived from phosphatidic acid (PtdOH) resulting from phospholipase D

(PLD) activation (21) . Under these conditions, the primary alcohol, butan-1-ol, traps the phosphatidyl-moiety generated by PLD-mediated hydrolysis of phosphatidylcholine as phosphatidylbutanol (PtdBut): PtdBut is not a substrate for the enzyme, phosphatidic acid phosphohydrolase, which converts PtdOH to DAG (21). FcγRI-coupled DAG was indeed shown to be derived from PtdOH generated by phospholipase D activation as pretreating cells with 0.3% butan-1-ol completely abolished the receptor stimulated rise in mass levels of DAG (Figure 2B).

Activation of phosphatidylcholine-specific phospholipase D (PtdCho-PLD) following aggregation of FcγRI was demonstrated by the definitive transphosphatidylation assay (21). These experiments showed that aggregation of Fcγ receptors in IFN-γ primed, [^{3}H]palmitate-labelled cells stimulated activation of PtdCho-PLD, as evidenced by substantial generation of [^{3}H]PtdBut, in the presence of butan-1-ol, over a 30 min time course (Figure 2C). Higher concentrations of butan-1-ol (1%) resulted in no further increase in measured [^{3}H]PtdBut compared to cells incubated with 0.3% butan-1-ol indicating that all the phosphatidyl moiety generated by PtdCho-PLD is trapped at the lower concentration of primary alcohol. Lower concentrations of butan-1-ol (0.1%) resulted in less measurable [^{3}H]PtdBut. Thus, the optimal concentration of butan-1-ol to trap the phosphatidyl moiety is 0.3% (Figure 2D). The specificity of the measurement was confirmed using butan-2-ol which is unable to trap the phosphatidyl moiety generated by PtdCho-PLD. No [^{3}H]PtdBut could be detected in cells preincubated with butan-2-ol even at 1% preincubation.

The increase in PtdCho-PLD activity following receptor aggregation was tyrosine kinase dependent as it was completely abolished by treating the cells

with genistein. Moreover, the concentration dependence of genistein-mediated inhibition of PtdCho-PLD (Figure 2E) showed a similar profile to that obtained for sphingosine kinase coupling (Figure 1C).

Thus, FcγRI is coupled through tyrosine kinases to the activation of PtdCho-PLD and sphingosine kinase

Legend for figure 3.2

A. Total InsPs accumulation over 20 mins following aggregation of Fcγ receptors. InsP accumulation over 20 mins was measured following the aggregation of human monomeric IgG (FcRI XL) in IFN-γ treated U937 cells in the presence of lithium chloride (10mM) and compared to accumulation following the specific aggregation of FcγRIIa (FcRIIa XL) in the cells treated in an identical way. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.

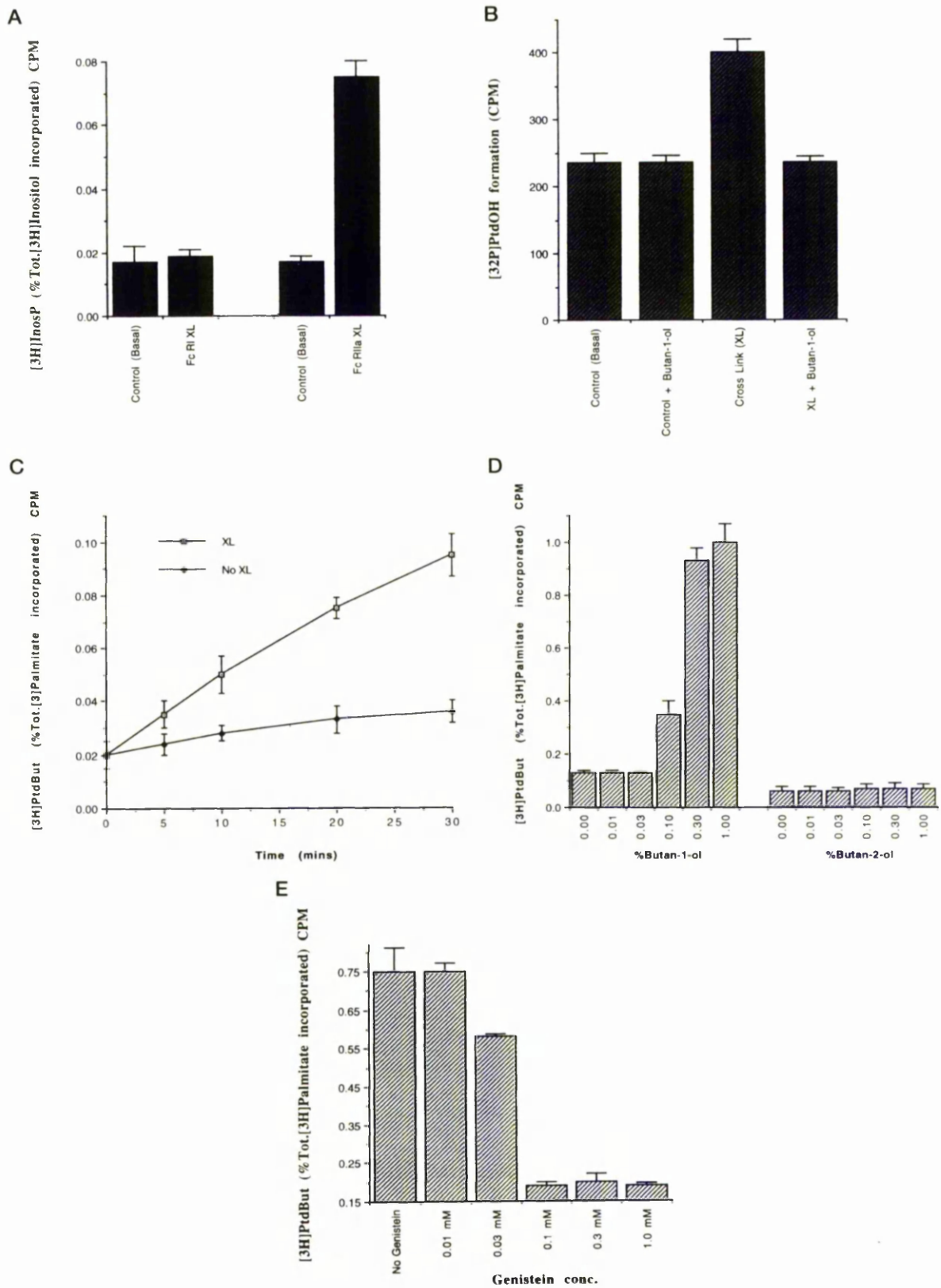
B. Accumulation of DAG over 20 mins following aggregation of Fcγ receptors. DAG was measured in cells 20mins after receptor crosslinking in cells preincubated with 0.3% butan-1-ol (XL + butan-1-ol) and compared to untreated cells (crosslink XL). The effect of butan-1-ol on basal levels was also measured (Control and control + butan-1-ol). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.

C. Activation of PtdCho-PLD following aggregation of Fcγ receptors. The time course of accumulation of [^{3}H]PtdBut was measured. The accumulation of [^{3}H]PtdBut in cells following addition of crosslinking antibody (XL) was compared to cells loaded with monomeric IgG but the crosslinking antibody was omitted (No XL). Cells were harvested at 5, 10, 20 and 30 mins. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.

D. Effect of varying concentrations of butan-1-ol and butan-2-ol on measurement of [^{3}H]PtdBut. Cells were pre-incubated with either butan-1-ol (0.03, 0.2, 0.3 and 1.0%) or butan-2-ol over the same range and the total [^{3}H]PtdBut. accumulation over 20 mins following addition of the crosslinking antibody was measured. Results shown are the mean ± S.D. for triplicate measurements at each time point.The results shown are typical from three separate experiments.

E. Activation of PtdCho-PLD following aggregation of Fcγ receptors. Accumulation of [^{3}H]PtdBut was measured, in cells 20 mins after aggregation of FcγRI. The activity was compared to cells pretreated with genistein for 30 mins and the effect of varying concentrations of genistein (0.01, 0.03, 0.1, 0.3, 1.0 mM) was measured. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.

Figure 3.2 Phospholipase D and not phospholipase C is activated by aggregation of FcγRI in IFN-γ primed cells.



3.4.3 Activation of sphingosine kinase is downstream of phospholipase D activation

To assess the relative relationship of activation of PtdCho-PLD and sphingosine kinase, studies were initially undertaken to explore comparative kinetics of activation and use of selective inhibitors. However, comparison of the relative kinetics was complicated by the difference in the assay characteristics for measuring sphingosine kinase and phospholipase D. Thus, sphingosine kinase is measured as an in vitro kinetic kinase assay whereas the assay for phospholipase D relies on the accumulation of a non-hydrolysable product. The difference in assay characteristics, therefore, precluded definitive early comparative time course analysis. The relationship of phospholipase D and sphingosine kinase activation was therefore addressed by examining selective inhibitors of the two enzymes.

To determine whether sphingosine kinase activation was upstream or downstream of phospholipase D, cells were pre-incubated with butan-1-ol or butan-2-ol for 20 mins before aggregation of FcγRI and the resultant sphingosine kinase activity compared to that of control cells. Pretreating cells with butan-1-ol (0.3%) completely abolished the normal sphingosine kinase response to FcγRI aggregation (Figure 3A). Consistent with these results, the rise in sphingosine-1-phosphate observed after aggregation of FcγRI was also blocked by pretreating cells with butan-1-ol (0.3%) (Figure 3B).

This data suggested that activation of sphingosine kinase is dependent on the activation of phospholipase D and generation of PtdOH. To assess this in detail, the effect on peak (30 secs after receptor aggregation) sphingosine kinase activity of preincubating cells with varying concentrations of butan-1-ol previously shown to influence phospholipase D was examined and these were compared to the same concentrations of butan-2-ol which does not influence phospholipase D (Figure 2D). Peak activity of sphingosine kinase

following FcγRI aggregation was abolished by incubating cells with either 0.3% or 1.0% butan-1-ol but was completely unaffected by preincubation with butan-2-ol even at the highest concentration (Figure 3C). Incubation of cells with a lower concentration of butan-1-ol (0.1%) partially inhibited peak sphingosine kinase activity. Of interest, 0.1% butan-1-ol resulted in lower concentrations of [^{3}H]Ptd-But (Figure 2D) suggesting that, at this concentration, butan-1-ol is only able to trap a proportion of the phosphatidyl moiety generated by phospholipase D and that as a result some phosphatidic acid may be produced.

This data using butan-1-ol indicates that phospholipase D is upstream of sphingosine kinase. Consistent with this observation, DHS, a competitive inhibitor of sphingosine kinase, had no effect whatsoever on phospholipase D activation at all concentrations examined even up to 100μM (Figure 3D). The potency of DHS on sphingosine kinase was measured directly; DHS at concentrations of 30μM and above completely abolished the peak sphingosine kinase activity observed after FcγRI aggregation; 10μM DHS inhibited peak shingosine kinase activity by about 75% (Figure 3E).

Taken together, this data clearly indicates that activation of sphingosine kinase is secondary to activation of phospholipase D and generation of PtdOH.

Legend for figure 3.3

A. Sphingosine kinase activity following FcγRI aggregation in cells treated with butan-1-ol. Cells were preincubated with 0.3% butan-1-ol and harvested at given times after aggregation of FcγRI for measurement of sphingosine kinase activity. Results (XL + butan-1-ol) were compared to control cells (crosslink XL). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.

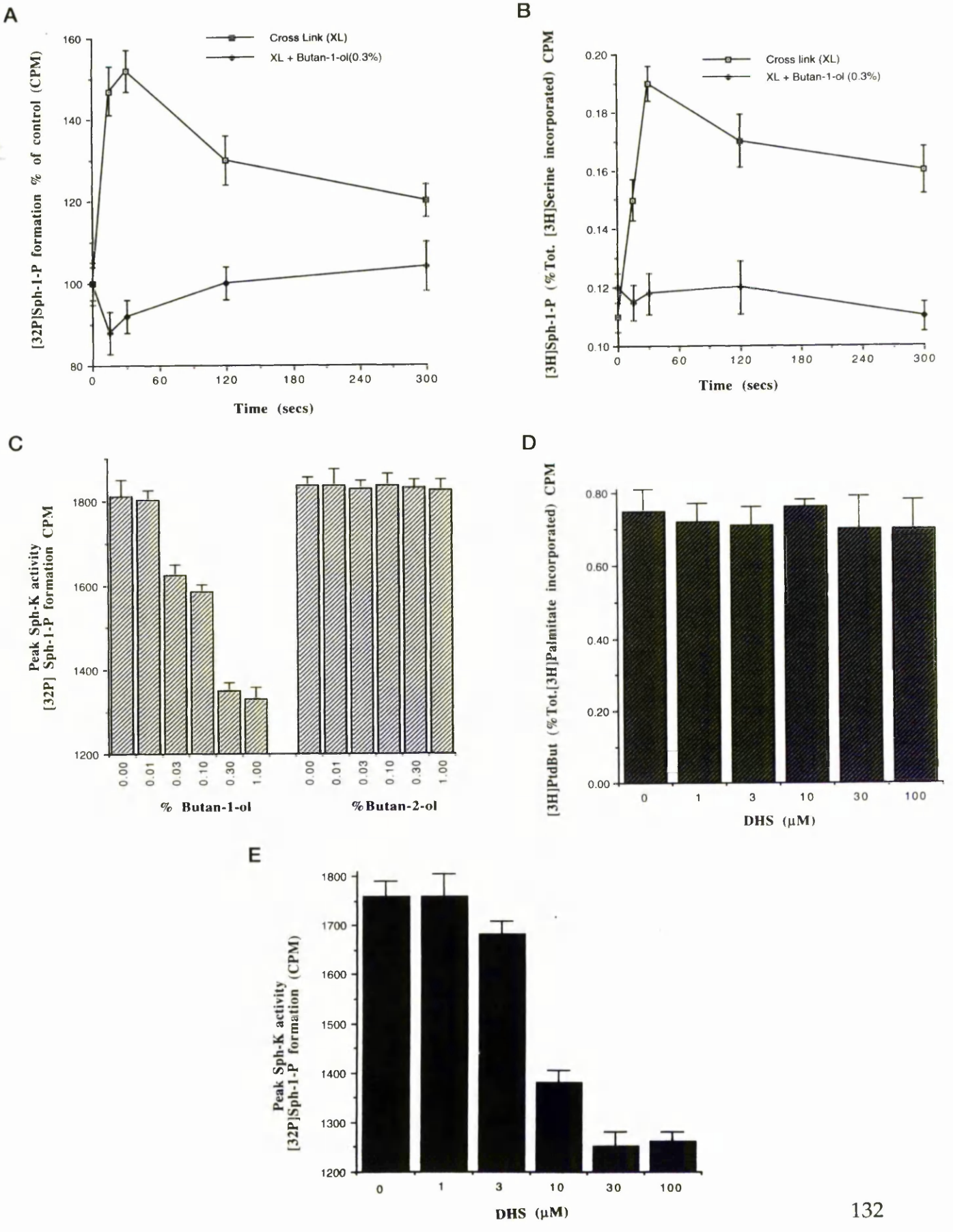
B. Sphingosine-1-phosphate concentrations in cells following receptor aggregation in cells treated with 0.3% butan-1-ol (XL+ butan-1-ol) compared to control untreated cells (crosslink XL). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.

C. Effect of varying concentrations of butan-1-ol or butan-2-ol on peak sphingosine kinase activity after FcγRI aggregation. Sphingosine kinase activity was measured in cells 30 secs after aggregating FcγRI in cells pre-treated for 30 mins with varying concentrations of butan-1-ol (0.03, 0.1, 0.3, 1.0%) and compared to cells incubated with butan-2-ol over the same concentration range. Results shown are the mean ± S.D. for triplicate measurements at each concentration. The results shown are typical from three separate experiments.

D. Effect of preincubating cells with varying concentrations of DHS on FcγRI-mediated phospholipase D activation. PLD activity was measured using the transphos-phatidylation accumulation assay (over 20 minutes) in cells following the aggregation of FcγRI in cells preincubated with varying concentrations of DHS (1, 3, 10, 30, 100μM). Results shown are the mean ± S.D. for triplicate measurements at each concentration. The results shown are typical from three separate experiments.

E. Effect of preincubating cells with varying concentrations of DHS on FcγRI-mediated sphingosine kinase activation. Sphingosine kinase activity was measured in cells 30 secs after aggregating FcγRI in cells preincubated with varying concentrations of DHS (1, 3, 10, 30, 100μM). Results shown are the mean ± S.D. for triplicate measurements at each concentration. The results shown are typical from three separate experiments.

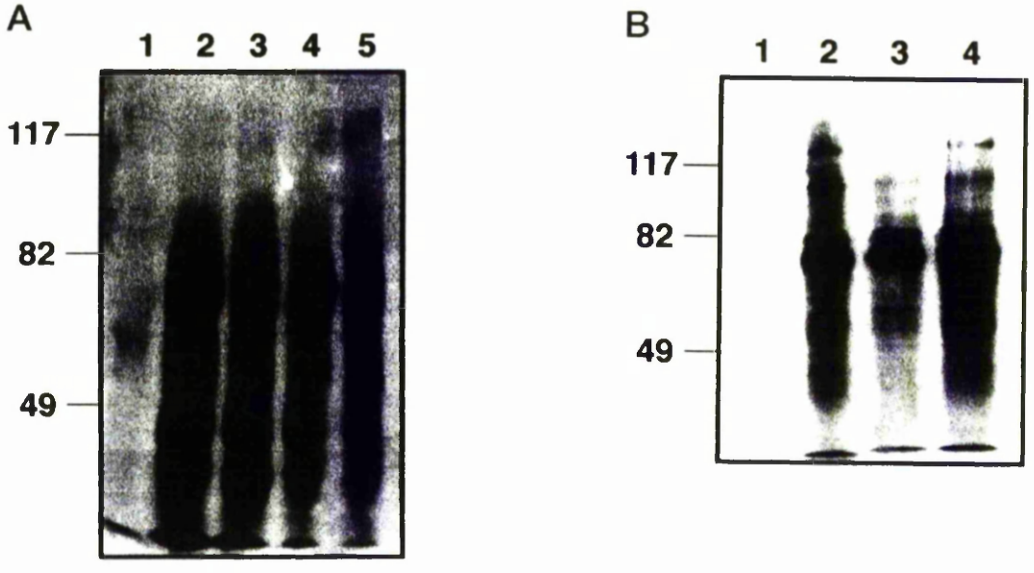
Figure 3.3 Sphingosine kinase activation is downstream of PtdCho-PLD activation.



3.4.4 Tyrosine phosphorylation is triggered promptly by aggregation of FcγRI and is upstream of both phospholipase D and sphingosine kinase

Tyrosine phosphorylation events were monitored in these cytokine primed U937 cells after aggregation of FcγRI by immunoprecipitating tyrosine phosphorylated proteins with a monoclonal antibody to phosphotyrosine. Consistent with other reports (5-7, 27, 28), addition of cross-linking antibody to form surface immune complexes resulted in the prompt appearance of a large number of tyrosine phosphorylated proteins. Preincubating cells with either butan-1-ol (0.3%) or DHS (25μM) did not influence the pattern of tyrosine phosphorylation (Figure 4), results consistent with our findings that both PtdCho-PLD (Figure 2C) and sphingosine kinase (Figure 1C) activation are downstream of tyrosine kinase activation.

Figure 3.4 Tyrosine phosphorylation occurs promptly after aggregation of FcγRI and is unaffected by inhibitors of phospholipase D and sphingosine kinase.



A. Western blot analysis of tyrosine phosphorylated proteins at given times after formation of surface immune complexes. Lane 1, cells loaded with monomeric IgG but no cross-linking antibody. Lane 2, cells loaded with monomeric IgG harvested immediately after addition of crosslinking antibody, Lane 3, cells harvested 1 minute after addition of crosslinking antibody, Lane 4, cells harvested 2 minutes after addition of crosslinking antibody and Lane 5, cells harvested 5 mins after addition of crosslinking antibody.

B. Tyrosine phosphorylation patterns in cells 2 mins after formation of immune complexes in cells treated with butan-1-ol (0.3%) (Lane 3) or DHS (25μM) (Lane 4) compared to control cells (Lane 2). Lane 1, cells loaded with monomeric IgG but no crosslinking antibody.

3.4.5 Activation of phospholipase D is necessary for both mobilisation of intracellular calcium and for trafficking of immune complexes for degradation

Release of intracellular stores of calcium by aggregation of FcγRI was significantly inhibited by pretreating the cells with 0.3% butan-1-ol (Figure 5A); thus, providing further support for the role of this pathway in mobilising calcium and the concept that PtdCho-PLD is upstream of sphingosine kinase. The possibility that butan-1-ol affected calcium mobilisation through non-specific effects was ruled out as subsequent addition of thapsigargin (250nM) resulted in a prompt response in cytosolic calcium. In addition, butan-1-ol had no effect on the $InsP_3$-dependent mobilisation of calcium following aggregation of the related low affinity receptor, FcγRIIa (Figure 5A). The difference in release of calcium after thapsigargin is not likely to be significant following manual injection as undertaken here. Although the speed of calcium release by thapsigargin can be influenced by a number of intracellular factors such as the amount of calcium in the stores, it is well recognised that there is considerable variability between runs for thapsigargin mediated calcium release and the largest influence is the rate of addition of thapsigargin and its mixing in the cuvette (29, 30).

As observed previously (17), internalisation of surface bound immune complexes is very rapid in IFN-γ treated U937 cells. The cell-associated counts plateau between 15 and 30 mins. However, over prolonged incubations, the internalised cell-associated counts were found to diminish gradually in the control cells such that, by 120 mins, approximately 50% of the cell associated counts had been lost (Figure 5B). This reduction was entirely matched by the appearance of counts in the culture media of the cells and these counts were not precipitable by trichloroacetic acid (TCA) (Figure 5B). The rate of

appearance of these TCA soluble counts in media is an indication of the rate of lysosomal degradation of the radiolabelled immune complexes (31) and is, therefore, a sensitive measure of the rate of intracellular trafficking of internalised immune complexes from endosomes to lysosomes.

Pretreatment of cells with butan-1-ol (0.3%) to inhibit PtdOH generation appeared to reduce to a small extent the initial phase of endocytosis (Peak % counts internalised control cells 93 $\pm$ 3 %; butan-1-ol treated cells 77 $\pm$ 2 %). Nonspecific effects of the alcohol were eliminated as butan-2-ol (0.3%) had no effect on the rate of endocytosis (Peak % counts internalised butan-2-ol treated cells 90 $\pm$ 3%). Following longer time intervals after internalisation of immune complexes, the amount of radiolabel trapped inside the cells gradually decreased in the untreated cells and in cells treated with butan-2-ol (0.3%) such that about 50% of the initial internalised radiolabel had been lost from the cells after two hours incubation. This loss of cell associated counts was entirely matched by the appearance in the cell supernatant of radiolabel in a form that was soluble in TCA. Thus, after 2 hours, 47 $\pm$ 2 % of the initial counts in the control cells appear as TCA soluble counts within the supernatant; this is a measure of trafficking of immune complexes for lysosomal degradation (31). Treatment of cells with butan-1-ol significantly inhibited trafficking of immune complexes for degradation. Thus, the rate of loss of cell associated (internalised) counts was significantly slowed in cells treated with butan-1-ol (after 2 hours incubation % counts remaining internalised - control cells 43 $\pm$ 5 %; butan-1-ol treated cells 63 $\pm$ 3 %). In addition, the rate of appearance of TCA soluble counts in the media over prolonged incubations was significantly slower for cells pretreated with 0.3% butan-1-ol compared to the control untreated cells or cells treated with butan-2-ol (Figure 5B). Thus, following 120 mins of incubation, only 19 $\pm$ 0.5% of counts appeared as TCA soluble counts in the media of cells treated with

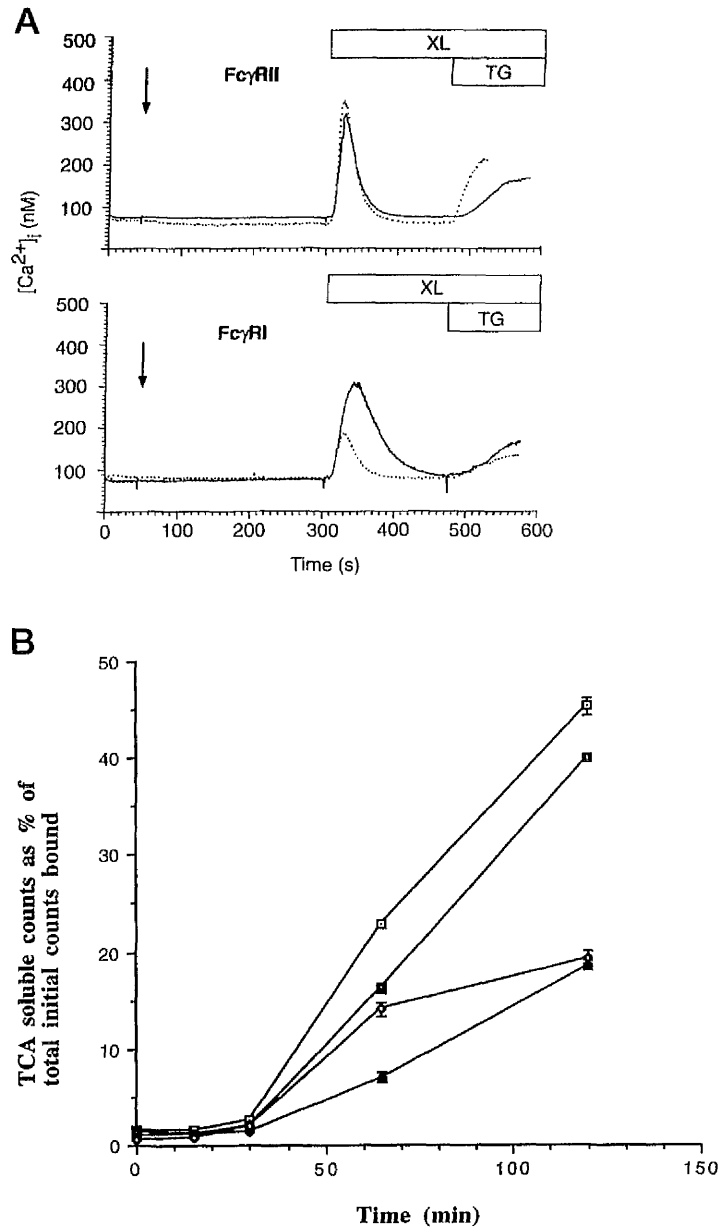
butan-1-ol in contrast to approximately 45 ± 1% for the control cells and 40 ± 0.4 % for cells treated with butan-2-ol.

Consistent with the biochemical data defining the signalling pathway, inhibition of sphingosine kinase with DHS (25μM) also significantly inhibited trafficking of immune complexes for lysosomal degradation (Figures 5B). Thus, activation of this intracellular signalling pathway involving phospholipase D and sphingosine kinase is required for the appropriate trafficking of internalised immune complexes along the degradative pathway.

Legend for figure 3.5

A. Butan-1-ol 0.3% inhibits the release of intracellular calcium stores initiated by FcγRI but has no influence on calcium release initiated by FcγRIIa. Cytosolic calcium concentrations were measured in cell populations prewarmed to 37°C following the aggregationof the Fc receptors.
Upper panel: calcium release in cells following the aggregation (XL) of FcγRI in cells pre-treated with 0.3% butan-1-ol (dotted line) was compared to untreated control cells (solid line). Thapsigargin (250nM) (TG) was added at the end of the experiment to ensure that calcium stores were intact. Typical trace from 5 separate experiments.
Lower panel: calcium release in cells following the specific aggregation (XL) of FcγRIIa in cells pre-treated with 0.3% butan-1-ol (dotted line) was compared to untreated control cells (solid line). Thapsigargin (250nM) (TG) was added at the end of the experiment to ensure that calcium stores were intact. Typical trace from 5 separate experiments.
B. Trafficking of immune complexes for degradation.
The rate of appearance in the culture medium of radiolabel as TCA soluble counts was measured in control cells and compared to cells preicubated with butan-1-ol (0.3%), butan-2-ol (0.3%) or DHS (25μM). Results shown for each time point are the counts in the incubation supernatant soluble in TCA expressed as a percentage of the total counts bound at time zero (17).
Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from two separate experiments.

Figure 3.5 Functional consequences of PtdCho-PLD and sphingosine kinase activation.



3.5 DISCUSSION

Taken together, this data indicates that FcγRI in cytokine primed U937 cells is coupled through tyrosine kinase activation to a novel pathway responsible both for mobilising calcium transients through an $InsP_3$-independent route and for trafficking internalised immune complexes for degradation. This novel pathway involves the activation of PtdCho-PLD, in the absence of measurable activation of phospholipase C, and this is upstream of activation of sphingosine kinase which generates sphingosine-1-phosphate.

Sphingosine -1-phosphate has been proposed previously to play a role in mobilising calcium from intracellular stores (32, 33, 34). However, this proposal has proved highly controversial due to the presence of extracellular G protein coupled receptors for sphingosine-1-phosphate (35, 36) which are able to mobilise calcium through conventional $InsP_3$ receptor-dependent pathways. The recent cloning of the SCaMPER receptor (37) provides additional evidence that sphingoid derivatives are able to engage intracellular receptors and effect calcium release from stores independently of $InsP_3$ generation. The data presented here provides evidence for specific immune receptor triggering of this pathway in myeloid cells. Thus, aggregation of FcγRI resulted in the rapid activation of sphingosine kinase and consequent cellular increases in sphingosine-1-phosphate concentrations. In these same cells, neither product of PLC activation could be detected; no accumulation of total InsPs could be measured even in the presence of lithium chloride to prevent breakdown. Moreover, the observed increase in DAG could be completely blocked by pretreatment of cells with butanol indicating PtdCho-PLD rather than PLC activation as the source of the DAG. In contrast, aggregation of an alternative immune receptor, FcγRIIa, on these cells, resulted in increases in both PLC-dependent DAG and InsP generation

indicating that this pathway is intact and functional in these cells and the assays used were potentially able to detect any such receptor triggered changes. Taken together, the data presented here suggests that the high affinity receptor, FcγRI, mobilises intracellular calcium through this sphingosine kinase-dependent, InsP3-independent pathway. In this respect FcγRI is behaving like the high affinity IgE receptor, FcεRI, in mast cells (15). Of interest, both these receptors use the same signal transducing molecule (γ chain) (10) to recruit soluble tyrosine kinases to mediate cellular activation. However, the mechanism of coupling of tyrosine kinases to sphingosine kinase activation following FcεRI aggregation in mast cells was, however, unclear (15). Here, we demonstrate that PtdCho-PLD is activated following aggregation of FcγRI in myeloid cells and that sphingosine kinase activation is dependent on PtdCho-PLD activation. The immediate product of PtdCho-PLD is phosphatidic acid and this is subsequently converted to DAG through the action of phosphatidic acid phosphohydrolase. Previous studies have shown that sphingosine kinase is activated by phosphatidic acid (38) and not by DAG (38) ; a product of both phospholipase D and phospholipase C. Our finding that sphingosine kinase is downstream of PtdCho-PLD is, therefore, consistent with this *in vitro* work. Moreover, both components of this novel FcγRI-coupled intracellular signalling pathway involving the sequential activation of PtdCho-PLD and sphingosine kinase depend on tyrosine kinase activation. This finding is consistent with previous *in vitro* studies demonstrating that v-Src can activate PLD (39).

Aggregation of FcγRI in myeloid cells triggers a number of effector functions. The novel intracellular signalling pathway demonstrated here appears to be functionally interactive/associated with these. Thus, previous studies have implicated phosphatidic acid in modulating neutrophil function, in particular by influencing the respiratory burst/NADPH oxidase cascade (40)

In this current study reported here, inhibiting this pathway at either the PtdCho-PLD and sphingosine kinase levels reduced or abolished the ability of this receptor to mobilise calcium from intracellular stores. In addition, inhibition of PtdCho-PLD significantly slowed the rate of trafficking of internalised immune complexes for degradation. Of interest, ADP-ribosylation factor plays a major role in regulating vesicular trafficking and this small molecular weight G protein has also been demonstrated to regulate phospholipase D activity (41). The finding that FcγRI is coupled to the release of intracellular calcium stores and vesicular trafficking via a novel pathway which does not use $InsP_3$ has profound implications for the development of strategies for therapeutic intervention against differential myeloid responses to immune complexes.

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CHAPTER 4

DIFFERENTIATION DEPENDENT SWITCH IN PKC ISOENZYME ACTIVATION BY FcγRI THE HUMAN HIGH AFFINITY RECEPTOR FOR IMMUNOGLOBULIN G

4.1 ABSTRACT

Aggregation of receptors for the constant region (Fc) of immunoglobulin G on myeloid cells results in endocytosis or phagocytosis and cellular activation. Previous work has shown, using the cell line U937, that the high affinity receptor, FcγRI, activates alternate intracellular signalling pathways depending on the cell differentiation state which results in a marked change in the nature of calcium transients within the cell. Here, we show that protein kinase C is activated in both IFN-γ primed and dbcAMP differentiated cells but that the nature of the particular isoenzymes recruited differ. Thus, in IFN-γ primed U937 cells, FcγRI aggregation results in an increase of PKC activity which is essentially calcium independent resulting from the translocation to the membrane of the novel PKCs, δ and ε, together with the atypical PKC ζ. However, in cells differentiated to a more macrophage phenotype, all PKC enzyme activity after receptor aggregation is calcium dependent. Consistent with this finding, the isoenzymes translocated to the nuclear-free membrane fraction are the conventional PKCs α, β, and γ; results consistent with our previous finding that FcγRI couples to phospholipase C in such dbcAMP-differentiated cells. Thus, the nature of PKC isoenzyme activated following FcγRI aggregation is defined by differentiation. The calcium dependence of the

PKC isoenzyme is consistent with the duration of calcium transients previously reported in the two differentiation states.

4.2 INTRODUCTION

Binding of the constant region (Fc) of immunoglobulin G (IgG) to cell surface receptors (FcγR) on leukocytes provides a pivotal link between the humoral and cellular arms of the immune system (see reviews 1–3). Three different classes of Fcγ receptors have been defined; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (see reviews 4–6). Of these, the human high affinity receptor, FcγRI, is an integral type I membrane glycoprotein (7) constitutively expressed on monocyte and macrophage cell types. On myeloid cells, aggregation of these receptors triggers a number of different effector functions including endocytosis of immune complexes or phagocytosis of opsonised particles. Fcγ receptor aggregation activates a repertoire of responses including degranulation and release of proteases, activation of the respiratory burst and release of cytokines. These can ultimately lead to targeted cell killing through antibody directed cellular cytotoxicity (ADCC) (8, 9) which is critically important for clearing virus infected cells and in cancer surveillance (10).

One feature of monocytes and macrophages is the heterogeneity of response to immune complex challenge using cells harvested under different conditions and different environments. Little is known about the signal transduction mechanisms underlying this or how they are modified as blood monocytes differentiate into tissue macrophages. Thus, to study early events in the FcγRI signalling pathway, we have used the human monocyte cell line, U937 (11), which constitutively expresses FcγRI and FcγRII and which allows controlled differentiation into a more macrophage cell type by treatment with dibutyryl cAMP (dbcAMP) (12). Previous work has shown that the nature of calcium transients markedly change as the cells become differentiated (13). Thus, a single spike in calcium is observed in response to Fcγ receptor aggregation of cells treated with interferon-γ whereas calcium oscillations are generated in cells differentiated to a more macrophage state by dbcAMP (14). This switch in

calcium signalling patterns is dictated by a switch in the intracellular signalling pathways activated by FcγRI in the two differentiation states (15). Thus, the calcium spike in IFN-γ treated cells results from the sequential activation of phosphatidylcholine phospholipase D (PtdCho-PLD) and sphingosine kinase (15, 16) whereas the calcium oscillations observed in dbcAMP differentiated cells are associated with activation of phosphatidylinositol 4,5 bisphosphate phospholipase C ($PtdInsP_2$-PLC) and subsequent generation of inositol 1,4,5 trisphosphate (InsP3). Both activation pathways generate diacylglycerol (17, 18) and thereby can activate protein kinase C (PKC) (18, 19) and this kinase has been shown to play an important role in mediating Fcγ receptor functions (20–23).

PKC isoforms, depending on their structure and cofactor regulation, are divided into three groups (24, 25): conventional (PKC α; βI; βII; and γ) which are calcium and diacylglycerol activated isoenzymes, novel (PKC δ; ε; η; μ and θ) which are calcium independent but diacylglycerol activated isoenzymes, and the atypical ones (PKC ζ; λ/ι) which do not require either calcium or diacylglycerol (24–28). Here, we show that the nature of the PKC isoenzymes activated by immune complexes differ in the two differentiation states. Thus, calcium dependent typical PKCs (α, β, and γ) were activated in dbcAMP differentiated cells whereas the calcium independent, novel PKCs, d and e, and the atypical isoenzyme z were activated in the cells primed with IFN-γ.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture.

U937 cells were cultured in a humidified atmosphere at 37°C, 6.8% CO_2 in RPMI 1640 medium (Gibco) supplemented with foetal calf serum (10%), glutamine (2 mM), penicillin (10 U ml^{-1}) and streptomycin (10 mg ml^{-1}). The cells were treated with IFN-γ (a gift from Bender Wein Ltd) (100 ng/ml) for 24 hours or dbcAMP (1mM) for 48 hours.

4.3.2 Analysis of PKC isoform expression.

U937 cells (10^7 cells), either IFN-γ primed or differentiated with dbcAMP, were harvested by centrifugation at 200g for 5 min and the cell pellet solubilised in 1 ml of lysis buffer (50 mM Tris, 150 mM sodium chloride, 2% NP40, 0.25% sodium deoxicholate, 1mM EGTA, 1 mM PMSF, 10mM sodium orthovanadate, 10μg/ml chymostatin, 10μg/ml leupeptin, 10μg/ml antipain and 10μg/ml pepstatin). Cell lysates were frozen in liquid nitrogen, then thawed and homogenised (15-20 strokes) on ice in a pre-cooled Dounce homogeniser. The cell lysates were centrifuged at 20,000 g for 15 minutes at 4°C and the supernatants containing total solubilised cellular protein was harvested and stored at -20°C. The amount of protein in supernatants was quantified using Bradford (BIO-RAD, UK) assay.

4.3.3 Aggregation of FcγRI.

Cells were harvested by centrifugation (200g for 5 min). After resuspension, the cells were incubated for 30 min on ice with 1μM human monomeric, polyclonal IgG (Serotec) to occupy surface FcγRI. Excess unbound ligand was removed by dilution and centrifugation. Cells were resuspended and ligand occupied receptors were then aggregated by addition of 1:50 dilution of F(ab)

goat anti-human IgG (Sigma) on ice. Cells were then warmed to 37°C for the times specified in the assays.

4.3.4 Cell Fractionation.

Following receptor cross-linking, cells were harvested at the specified times and subcellular fractions were prepared by a modification of methods previously described (29-31). Briefly, cells were harvested and resuspended in cold nuclear preparation buffer (10mM Tris-HCl, pH 7.4, 2mM magnesium chloride, 0.14M sodium chloride, 2% NP40, 0.25% sodium deoxycholate, 1mM EGTA, 1mM PMSF, 10mM sodium orthovanadate, 10µg/ml chymostatin, 10µg/ml leupeptin, 10µg/ml antipain and 10µg/ml pepstatin). Cells were frozen in liquid nitrogen, then thawed under running hot water, the nuclei released by 15-20 strokes of a pre-cooled Dounce homogeniser and centrifuged at 15,000g for 5 min. In order to examine the integrity of the nuclear membrane, the pellet was resuspended in 20µg/ml of propidium iodide and viewed by fluorescence microscopy. The supernatant was centrifuged at 100,000g and 4°C for 30 min, the pellet containing the membrane fraction resuspended in 200µl of the nuclear preparation buffer and stored at -20°C. The cytosol fraction, represented by the high speed supernatant, was also stored at -20°C. The amount of protein recovered in each fraction was quantified using the Bradford reagent system.

4.3.5 Gel Electrophoresis and Western Blots.

Proteins (50µg of whole cell lysate or 20µg of the membrane fraction), were resolved on 10% polyacrylamide gels (SDS-PAGE) under denaturing condition. The resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked by incubating overnight in PBS 5% non-fat milk, 0.1% Tween 20 buffer at 4°C. The membrane then was washed in PBS, 0.1%

Tween 20 and incubated individually with mouse monoclonal antibodies specific for each of human PKC isoforms (Transduction Laboratories) at dilutions as recommended by the manufacturer, in 5% non-fat milk/PBS/0.1% Tween 20 at room temperature for 4 hours. We have previously characterised the specificity of these PKC isoform specific antibodies (31). Following washing of the membranes, bands were visualised using ECL Western Blotting Detection System (Amersham International, Amersham, UK).

4.3.6 PKC enzyme activity assay.

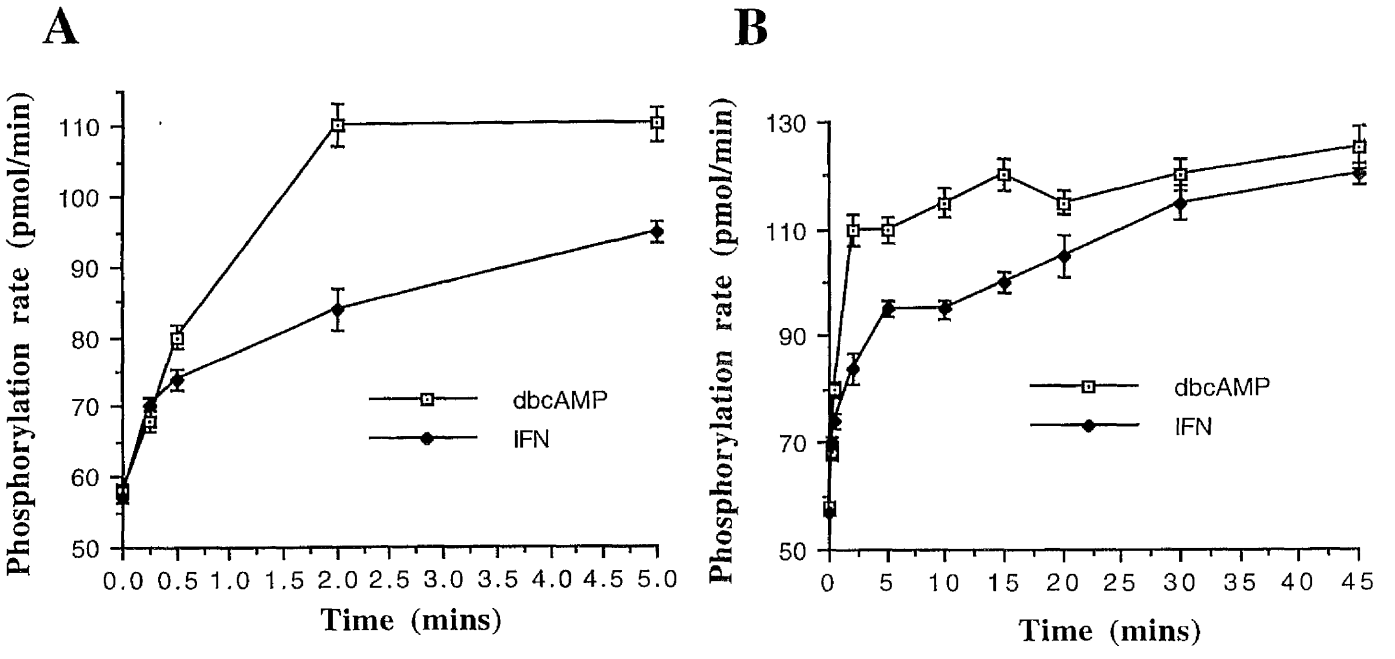
PKC assays were carried out using the Biotrak Protein Kinase C enzyme assay system (Amersham, UK). Briefly, the system is based upon the PKC catalysed transfer of the γ-phosphate group of adenosine-5'-triphosphate to a peptide substrate specific for PKC. U937 cells treated with IFN-γ or differentiated with dbcAMP were stimulated by FcγRI aggregation at the indicated times. Following stimulation proteins from whole cell lysates or fractionated, nuclear-free membrane samples, were partially purified by DEAE cellulose chromatography (Whatman DE52). PKC enzyme activity, from partially purified samples, was measured from whole cell lysate, or fractionated, nuclear-free, membrane samples in the presence of 1.5 mM calcium, or substituting calcium with 1.5 mM EGTA containing buffer.

4.4 RESULTS

4.4.1 Aggregation of FcγRI activates PKC in both IFN-γ primed and dbcAMP differentiated U937 cells.

The kinetics of activation of PKC was measured in cells primed with IFN-γ and compared to that observed for cells differentiated by dbcAMP. Resting PKC activity was identical in the two cell types and aggregation of FcγRI resulted in a prompt increase in PKC activity. Thus, 30 secs after receptor aggregation, PKC activity had doubled in both cell types (Figure 1A). In IFN-γ treated cells, this initial rapid rise in PKC activity was followed by a sustained steady increase which reached a plateau within 20 minutes and was maintained 45 minutes after receptor aggregation (Figure. 1B). In contrast, in dbcAMP differentiated cells, PKC activity increased very rapidly, such that peak PKC was achieved 2.5 minutes after receptor aggregation and this activity was then maintained over the subsequent 45 minutes (Figure. 1B). Although the time to reach plateau of PKC activity differed for IFN-γ primed and dbcAMP differentiated cells, the plateau levels eventually reached in both cell types was very similar.

Figure 4.1 PKC activity after Fcγ receptor aggregation in IFN-γ–primed or dbcAMP-differentiated U937 cells.



PKC activity, measured as the phosphorylation rate (pmol/min), in whole cell lysates.

A. PKC activity following FcγRI aggregation, (short, up to 5 min, time course) in dbcAMP differentiated (dbcAMP) and IFN-γ primed (IFN-γ) U937 cells. The data are the mean ± the standard deviation of triplicate measurements for each time point and are representative of four separate experiments.

B. PKC activity following FcγRI aggregation (long, up to 45 min, time course) in dbcAMP differentiated (dbcAMP) and IFN-γ primed (IFN-γ) U937 cells. The data are the mean ± the standard deviation of triplicate measurements for each time point and are representative of four separate experiments.

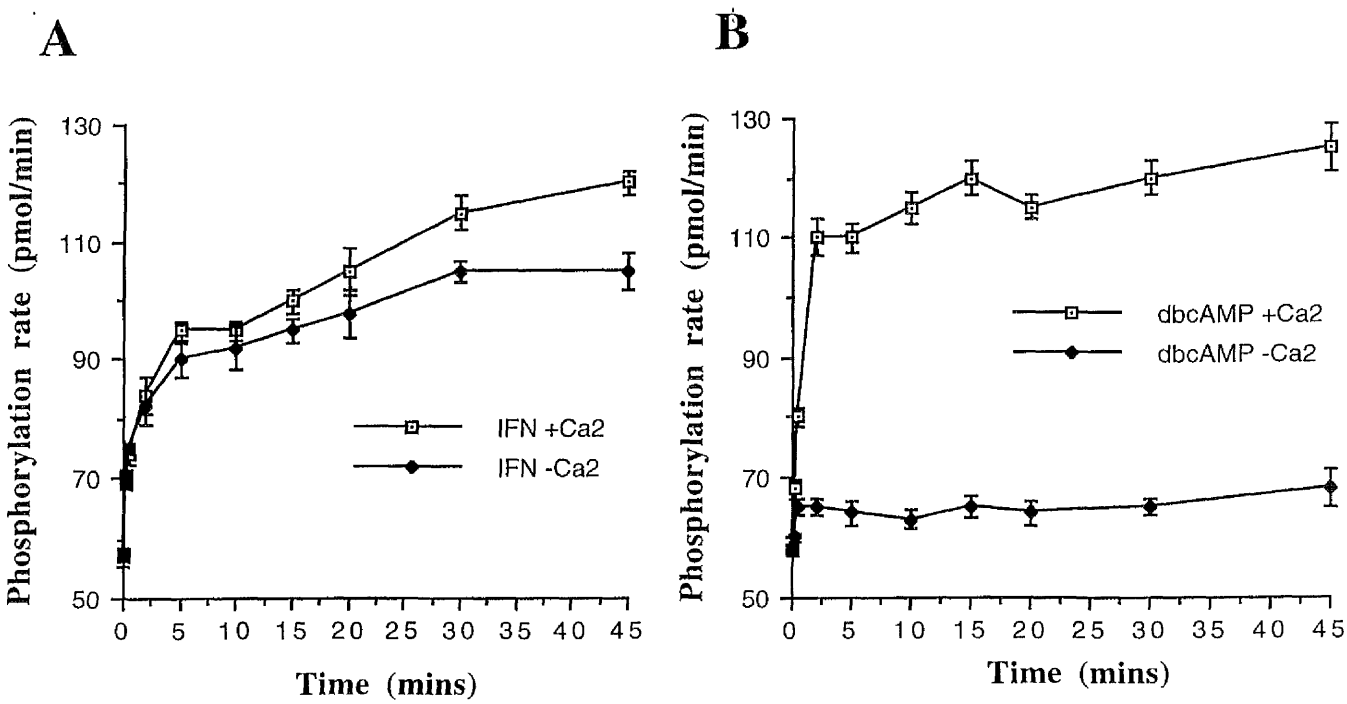
4.4.2 FcγRI aggregation results in calcium dependent or independent PKC enzyme activity depending on cell differentiation.

Previous work has shown that the nature of calcium transients generated following FcγRI aggregation markedly change following differentiation of the cells (12, 13). Since calcium is an important co-factor for PKC activity, we next investigated the calcium dependence of the observed PKC activity.

In IFN-γ primed cells, the increase in PKC activity over the first 20 minutes after receptor aggregation was unaffected by the withdrawal of calcium from the assay (Figure 2A). At the later time points (30 mins and 45 mins), addition of calcium resulted in a small increase in measured PKC activity. This data indicates that over the first 20 minutes after receptor aggregation, calcium independent novel or atypical PKCs account for the vast majority of the PKC activation observed in these cells. The calcium dependent classical PKCs only contribute to the total PKC activation in the later stages following receptor aggregation and even here they are only a minor component.

In contrast in dbcAMP differentiated cells, withdrawal of calcium from the assay completely abolished any increase in measurable PKC activity at all time points. Thus, in these cells, aggregation of FcγRI results in the activation of calcium dependent, typical PKCs and there is little contribution of calcium independent novel or atypical PKCs (Figure 2B).

Figure 4.2 Calcium dependence of PKC activity after Fcγ receptor aggregation in IFN-γ–primed or dbcAMP-differentiated U937 cells.



PKC activity, measured as the phosphorylation rate (pmol/min), in the presence or absence of calcium in the assay buffer.

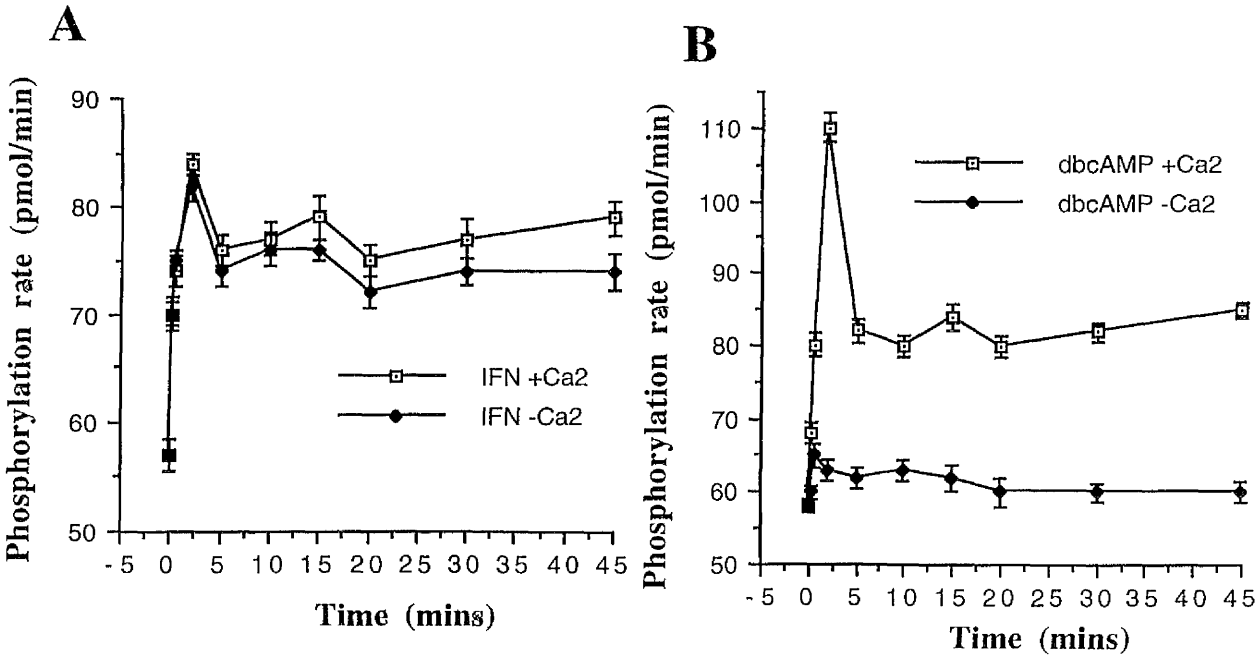
A. PKC activity following FcγRI aggregation in IFN-γ primed U937 cells in the presence of 1.5 mM calcium (IFN +Ca2) or substituting calcium with 1.5 mM EGTA (IFN –Ca2) in the assay buffer. The data are the mean ± the stardard deviation of triplicate measurements for each time point and are representative of three separate experiments.

B. PKC activity following FcγRI aggregation in dbcAMP differentiated U937 cells in the presence of 1.5 mM calcium (dbcAMP +Ca2) or substituting calcium with 1.5 mM EGTA (dbcAMP –Ca2) in the assay buffer. The data are the mean ± the stardard deviation of triplicate measurements for each time point and are representative of three separate experiments.

4.4.3 FcγRI aggregation results in PKC translocation to membranes.

PKC activation is associated with its translocation to the plasma membrane (24, 25, 32). PKC activity in the nuclear-free membrane fraction was therefore measured together with the calcium dependence of this activity in IFN-γ primed and dbcAMP differentiated cells following aggregation of FcγRI. In IFN-γ treated cells, membrane associated PKC activity increased very rapidly after receptor aggregation. An increase was observed within the first 15 seconds and reached maximal activity at 2 minutes; thereafter being sustained for 45 minutes (Figure 3A). Withdrawal of calcium from this assay had no effect on the PKC activity indicating that the translocated PKCs were predominantly calcium independent. In contrast, in dbcAMP differentiated cells, all the membrane associated PKC activity was dependent on the presence of calcium in the assay indicating that the PKCs translocated to the membrane are the conventional calcium dependent PKCs. In these cells, receptor aggregation also resulted in a very rapid increase in PKC activity in the membrane fraction. However, in contrast to the cells primed with IFN-γ, this membrane associated PKC activity peaked 2.5 minutes after receptor aggregation and then fell rapidly to achieve a new plateau level of activity that was maintained over the subsequent 45 minutes (Figure 3B).

Figure 4.3 PKC activity translocated to the nuclear-free membrane fraction and its calcium dependence after Fcγ receptor aggregation in IFN-γ–primed or dbcAMP-differentiated U937 cells.



A. PKC activity following FcγRI aggregation in IFN-γ primed U937 cells in the nuclear-free membrane fraction and in the presence of 1.5 mM calcium (IFN +Ca2) or substituting calcium with 1.5 mM EGTA (IFN –Ca2) in the assay buffer. The data are the mean ± the standard deviation of triplicate measurements for each time point and are representative of three separate experiments.

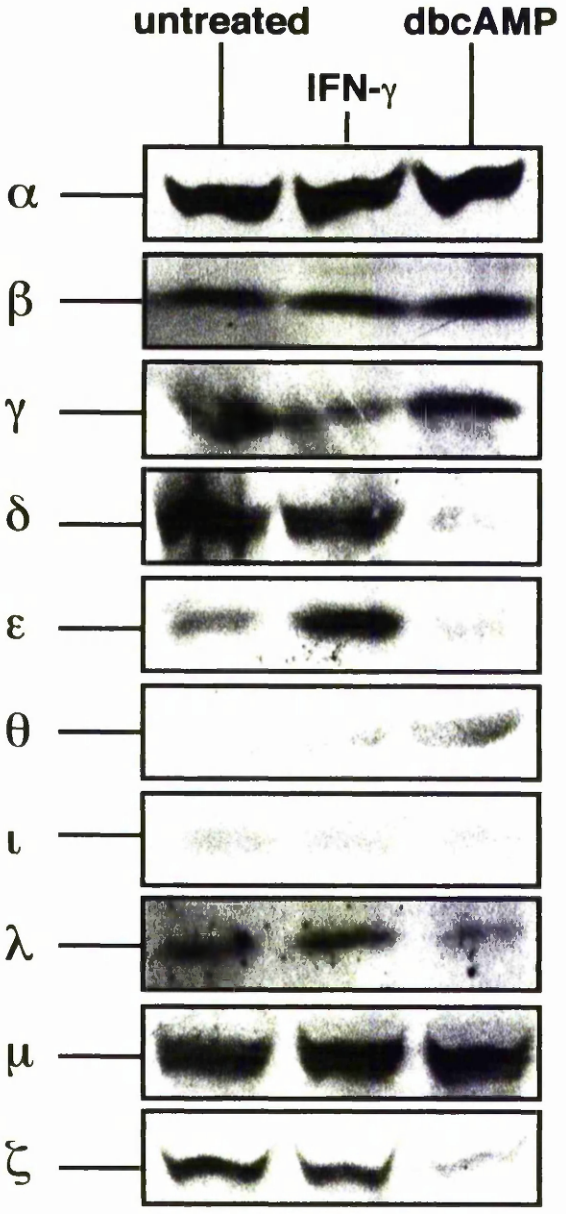
B. PKC activity following FcγRI aggregation in dbcAMP differentiated U937 cells in the nuclear-free membrane fraction and in the presence of 1.5 mM calcium (dbcAMP +Ca2) or substituting calcium with 1.5 mM EGTA (dbcAMP –Ca2) in the assay buffer. The data are the mean ± the standard deviation of triplicate measurements for each time point and are representative of three separate experiments.

4.4.4 PKC isoenzyme expression is regulated by differentiation.

Several PKC isoenzymes, from the three defined groups, have been shown to be expressed in monocytes and macrophages (21, 33). However, changes following differentiation are still unclear. The findings reported here indicate that calcium independent PKCs are preferentially activated in IFN-γ treated cells compared to dbcAMP differentiated cells where calcium dependent PKCs are activated. To determine whether this change in isoenzyme recruitment following FcγRI aggregation reflected a change in the relative expression of the various PKCs, cell lysates were probed with monoclonal antibodies specific for each enzyme for cells primed with IFN-γ or differentiated with dbcAMP and the patterns compared to that for untreated, resting cells.

Western blot analysis showed that in untreated U937 cells PKC α, β, γ, δ, ε, θ, μ, ι/λ, and ζ, are all expressed to varying degrees (Figure 4 lane 1). Treatment of the cells with IFN-γ resulted in an increase in PKC δ, ε, μ, ι/λ, and θ. PKC α, and ζ remained unchanged and a reduction in expression of β, and γ isoenzymes was observed (Figure 4 lane 2). In contrast dbcAMP differentiation resulted in upregulation of PKC α, β, and γ, and downregulation of PKC δ, ε, ι/λ, and ζ. PKC μ and θ remained unchanged by dbcAMP treatment of U937 cells (Figure 4 lane 3).

Figure 4.4 Analysis of the different PKC isoenzymes expressed in: U937 cells; IFN-γ-primed U937 cells; and dbcAMP-differentiated U937 cells.



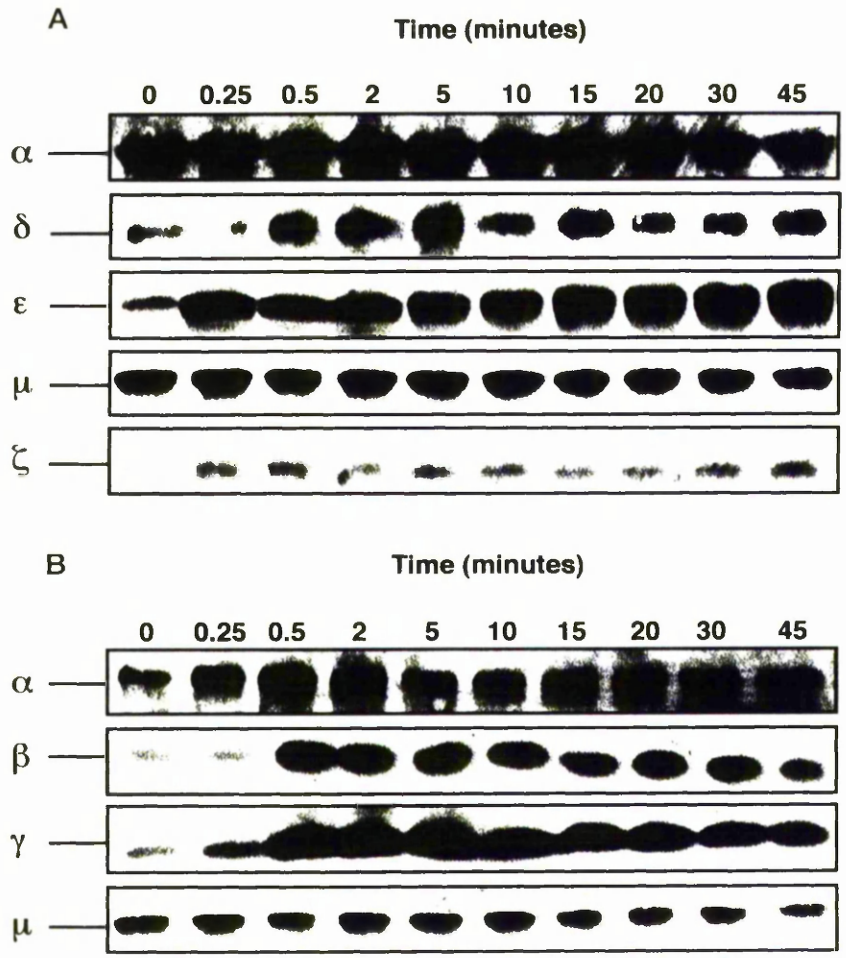
Cells were cultured in their normal growth media (untreated), or in media containing IFN-γ (100 nγ/ml) for 24 hr (IFN-γ), or in media containing dbcAMP (1mM) for 48 hr (dbcAMP). Samples from cell lysates were subjected to SDS/PAGE and Western blotted. The blots were incubated individually with mouse monoclonal antibodies specific for each of human PKC isoenzymes (α, β, γ, δ, ε, θ, ι/λ, μ, ζ). Bands were visualised by ECL (Amersham).

4.4.5 Differentiation-dependent differential translocation of PKC isoenzymes to the membrane fraction following FcγRI cross-linking.

To determine whether aggregation of FcγRI results in differential activation of particular isoenzymes in IFN-γ primed cells compared to dbcAMP differentiation, specific translocation of individual PKC was investigated. In IFN-γ primed cells, the novel PKCs δ and ε and atypical ζ isoenzymes were translocated to the membrane fraction following FcγRI cross-linking. However, all of the conventional PKC α appeared to be membrane bound in non-stimulated IFN-γ-primed cells and thus translocation could not be assessed (Figure 5A). In contrast, in dbcAMP differentiated cells, only the conventional PKCs α, β, γ were translocated to the membrane fraction in response to FcγRI cross-linking.

The novel PKC μ , which contains a putative transmembrane domain, is as expected, membrane bound in both IFN-γ primed and dbcAMP differentiated cells even in the absence of receptor aggregation and thus translocation could not be assessed (Figure 5B). No other PKC isoenzymes could be detected in the nuclear-free membrane fraction.

Figure 4.5 Protein analysis of the different PKC isoenzymes translocated to the nuclear-free membrane fraction following FcγRI aggregation in IFN-γ primed and dbcAMP differentiated U937 cells.



A. Western blots analysis of the different PKC isoenzymes translocated to the nuclear-free membrane fraction of IFN-γ primed U937 cells following FcγRI aggregation time course. The blots were incubated individually with mouse monoclonal antibodies specific for each of human PKC isoenzymes (α, β, γ, δ, ε, θ, ι/ λ, μ, ζ). PKC isoenzymes found to translocate δ, ε, ζ. PKCs α and is membrane bound throughout the experiment.

B. Western blots analysis of the different PKC isoenzymes translocated to the nuclear-free membrane fraction of dbcAMP differentiated U937 cells following FcγRI aggregation time course. The blots were incubated individually with mouse monoclonal antibodies specific for each of human PKC isoenzymes (α, β, γ, δ, ε, θ, ι/ λ, μ, ζ). PKC isoenzymes found to translocate α, β, γ. PKC μ is membrane bound throughout the experiment.

4.5 DISCUSSION

Endocytosis of immune complexes by FcγRI has been shown here to cause a prompt and sustained activation of PKC in both IFN-γ primed U937 cells and cells differentiated to a more macrophage phenotype by dbcAMP. However, the precise nature of the PKCs activated by receptor aggregation differs. Calcium independent PKC isoenzymes were activated in IFN-γ primed cells whereas, following differentiation, calcium dependent PKCs were the major activated form. Although IFN-γ and dbcAMP modified the relative expression levels of the various PKC isoenzymes, all the major species were still present in lysates of both cell types and thus, the changes in expression cannot account for the fundamental switch in PKC isoenzyme activation following receptor aggregation. This observation implies that FcγRI is able to recruit and specifically activate different isoenzymes depending on the differentiation state of the cell and likely results from the switch in signalling pathways activated by FcγRI following differentiation. This switch dictates the duration of calcium transients in these cells and it is noteworthy that the calcium dependence of the PKCs activated by FcγRI reflects this change.

Previous work has shown that, in these two differentiation states, FcγRI is coupled to the activation of distinct intracellular signalling pathways. FcγRI is able to switch between these two activation pathways as its cytoplasmic tail contains no signalling motif but, rather, the receptor must recruit an accessory molecule to activate tyrosine kinases. Thus, in IFN–γ primed cells, FcγRI acts through the γ chain and is coupled to the activation of PtdCho-PLD and sphingosine kinase. In these cells, changes in cytosolic calcium are transient as, following release from stores, calcium levels peak within 1 min and then fall to basal levels. Consistent with this, the PKC isoenzymes activated are

calcium independent and the predominant PKCs translocated to membranes are the isoenzymes δ, ε and ζ.

Novel PKCs are activated by diacylglycerol which is generated in this pathway by phosphatidic acid-phosphohydrolase (PPH) converting phosphatidic acid, the immediate product of PtdCho-PLD, to diacylglycerol. However, the diacylglycerol species produced by this pathway differs from that generated by $PtdInsP_2$-PLC and recent evidence suggests that the diacylglycerol produced by PtdCho-PLD and PPH is not able to activate PKC (34). Atypical PKCs are not activated by either calcium or diacylglycerol. However, phosphatidic acid itself has been shown to activate specifically PKC ζ; thus, providing a potential link between the novel intracellular signalling pathway we have previously defined in IFN-γ primed U937 cells following FcγRI aggregation and the pattern of PKC activation observed. Our recent work has shown that aggregation of FcγRI also activates PI3-kinase in IFN-γ treated cells (35) and that PI3-kinase is upstream of phospholipase D activation. This finding provides an explanation for the observations reported here as both novel and atypical PKCs are activated by second messengers generated by PI3-kinase (36--39).

In IFN-γ primed cells, FcγRI recruits the γ chain to act as its accessory molecule for signal transduction (40, 41, 15). The γ chain acts as a signal transducing accessory molecule for a number of receptors including the high affinity IgE receptor, FcεRI in mast cells (42). In cells expressing FcεRI, endocytosis of this receptor has been correlated with threonine phosphorylation of the γ chain and this phosphorylation was attributed specifically to PKC δ activation (43). Of interest, PKC δ was physically associated with the FcεRI receptor complex in particular with the β chain of the receptor complex. Although similar phosphorylation of threonine residues of the γ chain has been shown following aggregation of FcγRI (44), PKC δ immunoreactivity could not be

demonstrated in FcγRI immunoprecipitates either before or after receptor aggregation by immune complexes (data not shown). This may result from the lack of expression of the FceRI - b chain in myeloid cells.

In contrast to IFN-γ primed cells, differentiation of U937 cells by dbcAMP to a more macrophage phenotype results in a switch in the intracellular signalling pathways activated following FcγRI aggregation (15). Thus, PtdInsP_2 PLC and not PtdCho-PLD is activated with the subsequent generation of diacylglycerol and InsP_3. Associated with this, the calcium transients are prolonged as store dumping is followed by activation of I_{CRAC} and significant calcium entry (45, 46). Consistent with this, the PKCs activated are the conventional type being calcium dependent and, specifically, the isoenzymes α, β and γ are translocated to membranes. The switch observed in activation pathway results from a switch in the accessory molecule used by FcγRI as in the dbcAMP differentiated cells, signalling by FcγRI appears to be mediated by FcγRIIa (15).

Activation of PKC is well established as essential in mediating phagocytosis in myeloid cells (20–23). The switch in calcium dependence of the activated PKC isoenzymes in the two cell types provides an explanation for the observation that phagocytosis mediated by FcγRI is calcium independent whereas phagocytosis mediated by FcγRII is calcium dependent (20). In dbcAMP, FcγRII is used as the signal transducing molecule (15). In these cells, only calcium dependent PKCs are activated. Buffering of intracellular calcium would therefore abolish PKC activation and thereby prevent phagocytosis. In IFN-γ primed cells, FcγRI uses the γ chain for signal transduction and, under these circumstances, calcium independent PKCs are activated. Buffering intracellular calcium will therefore have no effect on PKC activation.

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CHAPTER 5

AGGREGATION OF THE HUMAN HIGH AFFINITY IMMUNOGLOBULIN G RECEPTOR (FcγRI) ACTIVATES BOTH TYROSINE KINASE AND G-PROTEIN COUPLED PHOSPHOINOSITIDE 3-KINASE ISOFORMS

5.1 ABSTRACT

Phosphoinositide 3-kinases (PI3-kinases) play an important role in the generation of lipid second messengers and the transduction of a myriad of biological responses. Distinct isoforms have been shown to be exclusively activated either by tyrosine kinase coupled- or G-protein-coupled receptors. We show here, however, that certain non-classical receptors can couple to both tyrosine kinase- and G-protein-dependent isoforms of PI3-kinase: thus, aggregation of FcγRI, the human high affinity IgG receptor, on monocytes unusually leads to activation of both these types of PI3-kinase. Following aggregation of FcγRI, PIP_3 levels rise rapidly in IFN-γ primed cells reaching a peak within 30 secs. Moreover, and in contrast to the situation observed following stimulation of these cells with either insulin or ATP, which exclusively activate the tyrosine kinase- and G-protein-coupled forms of PI3-kinase, respectively, PIP_3 levels remain elevated even 2 mins after receptor aggregation. We show here that whilst the initial peak results from transient activation of the p85-dependent p110 isoform of PI-3kinase, presumably through recruitment of tyrosine kinases by the γ chain, the later sustained rise of PIP_3 results from activation of the G-protein βγ subunit-sensitive isoform, p110γ. This is the first evidence that receptors which lack an intrinsic signalling motif, such as FcγRI, can recruit both tyrosine kinase and G-protein-coupled intracellular signalling molecules and thereby initiate cellular responses.

5.2 INTRODUCTION

Fc receptors (FcγRs) specific for IgG are expressed on the surface of many different cell types of the immune system and play an important role in linking the cellular and humoral arms of the immune response (1-3). On myeloid cells aggregation of FcγRs leads to a number of cellular responses including the internalisation of immune complexes by endocytosis or opsonised particles through phagocytosis, degranulation and the release of proteases, activation of the respiratory burst, and the release of cytokines. These processes can lead to targeted cell killing through antibody directed cellular cytotoxicity (ADCC) (4, 5) which is important for the clearance of virus infected cells and in cancer surveillance (6).

Three classes of FcγRs have been identified and cloned in mammals (FcγRI, FcγRII and FcγRIII), each of which has a variety of isoforms with differing affinities for IgG and tissue distributions (1, 2). FcγRI is the human high affinity receptor (7) and although its cytoplasmic tail contains no obvious signalling motif, FcγRI has been shown to associate physically with the ITAM-containing γ chain (8, 9) and FcγRI cross-linking results in signal transduction as evidenced by tyrosine phosphorylation events (10-13) and tyrosine kinase dependent calcium transients (14, 15).

Phosphoinositide 3-kinases (PI3-kinases) catalyse the phosphorylation of inositol phospholipids at the 3-position of the inositol ring (16). The phospholipids produced by the actions of these enzymes; PtdIns(3)P, PtdIns(3,4)P_2 (PIP_2) and PtdIns(3,4,5)P_3 (PIP_3), act as second messengers which are increasingly implicated in regulating a number of cellular responses. For example, PI3-kinase activity has been implicated as being involved in insulin-induced glucose transport (17), platelet-derived growth factor- and insulin-induced actin rearrangement (18, 19) and in the regulation of neuronal survival (20).

There are three classes of PI3-kinases which are determined by their *in vitro* substrate specificities. Class I PI3-kinases phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2. This class of enzymes can be further subdivided into class IA and class IB. Class IA PI3-kinases consist of p110 catalytic subunits that are regulated by the SH2/SH3-domain containing p85 family of adaptor proteins and consist of the isoforms p110α, p110β, and p110δ (21-23). The p85 family of adaptor proteins (24-26) facilitate PI3-kinase interactions with other proteins through their SH2 and SH3 domains. Class IB consists of p110γ, an enzyme which associates with a p101 adaptor protein and is stimulated by G-protein βγ subunits (27-30). Class II PI3-kinases phosphorylate PtdIns and PtdIns(4)P but not PtdIns(4,5)P_2 (31, 32) and class III PI3-kinases have a substrate specificity restricted to PtdIns and are homologous to yeast Vps34p (vacuolar protein sorting defective) which is involved in the trafficking of proteins from the Golgi to the yeast vacuole (33-35).

Previous studies have shown that receptors that are coupled to tyrosine kinases, such as insulin, exclusively activate class IA PI3-kinases through the p85 adaptor molecule. In contrast, receptors that engage heterotrimeric G proteins, such as ATP, exclusively activate class IB PI3-kinases through the generation of βγ subunits. No crosstalk between the coupling of these two receptor to PI3-kinases has been shown. We report here the first example of a single receptor, FcγRI, which has the capacity to activate both class IA and class IB PI3-kinases.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Human IgG and sheep-anti-human IgG (γ chain specific) were obtained from Serotec. Potassium oxalate, and all phospholipids were from Sigma. [^{32}P]orthophosphate (HCl free) was obtained from Amersham. Wortmannin, lavendustin A and tyrphostins A23 and A25 were obtained from Calbiochem and genistein was from Sigma. Cells were treated with inhibitors at concentrations specified for 30 minutes at 37°C prior to receptor aggregation or stimulation.

Antisense oligonucleotides were purchased from Oswell DNA Services (Southampton, UK). Anti-p110γ oligonucleotides corresponded to the first 8 (24 mer) or 10 (30 mer) amino acids of p110γ. The sequences of these oligonucleotides were

5' CTGTTTATAGTTCTCCAGCTCCAT 3' and

5' CACGGGCTGTTTATAGTTCTCCAGCTCCAT 3'.

A control oligonucleotide (24 mer) of random sequence was also made, the sequence of which was

5' CTGGTGGAAGAAGAGGACGTCCAT 3'.

The 24 mers were capped at either end by phosphothiorate linkages (first two and last two linkages) whereas each linkage of the 30 mer consisted of a phosphothiorate. Cells were incubated with 10 μM of oligonucleotide for one hour prior to, and then for the duration of culture with IFN-γ.

5.3.2 Cell culture

U937 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 10 units/ml penicillin and 10 mg/ml of streptomycin at 37°C, 6.8% carbon dioxide in a water saturated atmosphere.

U937:Δp85 cells (a generous gift from L. Stephens, Barbraham Institute, Cambridge, UK) were similarly cultured, but in addition were cultured in the presence of 0.6 mg/ml G418 and 0.1 mg/ml hygromycin B (both from Calbiochem). Expression of Δp85 was induced with 15 mM IPTG, 5 nM PMA and 100 μM zinc chloride for 10 hours. All cells were primed with 200 ng/ml of IFN-γ (a kind gift from Bender Wein Ltd) for 24 hours prior to experimentation.

5.3.3 FcγRI cross-linking

Cells were harvested by centrifugation and then incubated at 4°C with 1 μM human monomeric IgG (Serotec) to occupy surface FcγRI. Excess unbound ligand was removed by dilution and centrifugation of the cells. Cells were resuspended in ice cold RPMI 1640/10 mM HEPES/0.1% BSA (RHB) and cross-linking antibody (goat anti-human IgG; 1:50 dilution) was added. Cells were then warmed to 37°C for the times specified in the assays.

5.3.4 Insulin and ATP stimulation

Cells were harvested by centrifugation, resuspended in ice cold RHB, and incubated with either 10 μg/ml insulin or 100 μM ATP. Cells were then warmed to 37°C for the times specified in the assays.

5.3.5 Pertussis Toxin activation and Culture

Pertussis toxin (90 μg/ml) was activated by incubation in 25 mM DTT for 30 min at 37°C. The concentration was then adjusted to 60 μg/ml with 75 mM Tris-HCl, pH 7.5 containing 1 mg/ml BSA. Pertussis toxin was then incubated overnight with cells at a final concentration of 1 μg/ml (36).

5.3.6 Measurement of Phosphoinositide 3-kinase activity

U937 cells ($2x10^7$ cells/ml) were treated with IFN-γ as described and harvested by centrifugation. Cells were then washed in phosphate free RPMI and resuspended in phosphate free RPMI containing 10% dialysed FCS. Cells were labelled with 500 μCi/ml $[^{32}P]PO_4$ for 90 min at 37°C. After labelling, cells were washed in ice cold RHB. Cells were stimulated with either insulin or ATP, or FcγRI cross-linked, and the reactions stopped at specified times with ice-cold PBS. Cells were permeabilised with methanol and the lipids extracted with chloroform. Aliquots from the organic phase were used to analyse total label incorporation whilst duplicate aliquots were dried down under vacuum, resuspended in chloroform: methanol (19:1), PIP_2 (10 μg/ml) standard added and spotted onto a silica TLC G60 plate (20 x 20 cm x 250 μm ; Whatman) precoated with potassium oxalate. Plates were developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14) and the standards visualised with iodine vapours. Radiolabelled bands were located by autoradiography or phosphorimaging, and the PIP_3 band was scraped into scintillation vials, scintillant added and the associated radioactivity determined by liquid scintillation counting. Quantification of labelled PIP_3 by phosphorimaging or scintillation counting always yielded the same ratio of PIP_3 generation.

5.3.7 Western blot

U937:Δp85 cells were induced to express Δp85 as described earlier. Non-induced or induced cells (10^7) were harvested by centrifugation and solubilised in sample buffer (37) containing 50 mM DTT. Samples were boiled for **15** minutes and run in a 10 % SDS-polyacrylamide gel (38). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2 μM pore size) as described (39). The blots were tested for the

overexpression of Δp85 by Western blotting with a polyclonal anti-p85α antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Western blots were developed using the ECL system (Amersham).

5.3.8 Immunoprecipitation

For immunoprecipitations, cells were lysed with ice-cold RIPA lysis buffer containing 1 mM PMSF, 1μg/ml CLAP (1μg/ml each chymostatin, leupeptin, antipain and pepstatin) 1 mM sodium orthophosphate and 1 mM sodium fluoride, for 30 min. Cellular debris was removed by centrifugation at 13,000 rpm for 15 min, and the cell lysates were precleared with protein A-agarose. Cell lysates were either incubated with 10 μg anti-p110 γ (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) followed by 10 μg protein A-agarose (Santa Cruz Biotechnology) or 10 μg of anti-p85-PI3 kinase conjugated to 10 μg of protein A-agarose (Upstate biotechnology, Lake Placid, NY) to immunoprecipitate p85 and p110γ.

For the studies of tyrosine phosphorylation of p85, IFN-γ-treated cells were loaded with human IgG and cross linked as described . Cells were harvested at specified times, lysed and p85 immunoprecipitated from the cell lysates by incubating 500 μl cell lysate with 10 μg anti-PI3 kinase/protein A-agarose overnight. Proteins were harvested by centrifugation of the agarose beads and dissociated from the beads by boiling in sample buffer (37) containing 50 mM DTT for 15 minutes and run in a 10 % SDS-polyacrylamide gel (38). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2 μM pore size) as described (39). The presence of tyrosine phosphorylated proteins was then detected by Western blotting with a monclonal anti-phosphotyrosine antibody (clone 4G 10; Upstate Biotechnology). Westerns were developed using the ECL system (Amersham).

For the quantitation of the effect of antisense oligonucleotides on p110γ levels, cells were incubated with the relevant oligonucleotides as described. Cells were harvested, lysed and p110γ immunoprecipitated from the cell lysates. Proteins were harvested and subjected to SDS-PAGE electrophoresis as above. Blots were probed for the presence of p110g and visualised by probing with radiolabelled (^{125}I) donkey anti-goat. Specific bands corresponding to p110γ were quantified using a phosphorimager (Fuji, FUJIX BAS 1000).

5.4 RESULTS

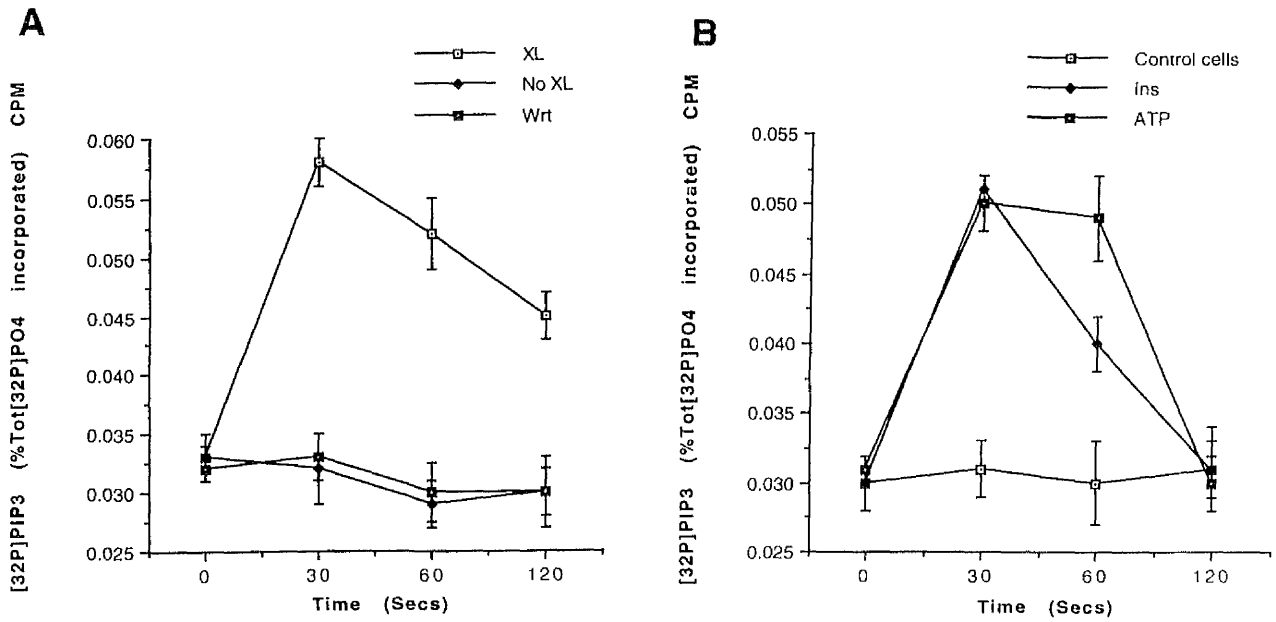
5.4.1 Cross-linking FcγRI stimulates phosphoinositide 3-kinase activity

Following the formation of surface immune complexes in interferon-γ (IFN-γ) primed U937 cells, the cellular levels of phosphatidylinositol 3, 4, 5-triphosphate (PIP_3) rose rapidly reaching a peak 30 seconds after receptor aggregation (Figure 1A). After this initial peak, PIP_3 levels were reduced but remained elevated about 2-fold above control (non-cross-linked cells) up to two minutes after cross-linking (Figure 1A). This 2 fold stimulation above basal levels was sustained and remained elevated 15 minutes after receptor cross-linking (data not shown). All of this stimulated increase in PIP_3 levels could be abolished by treating the cells before receptor aggregation with the PI3-kinase specific inhibitor wortmannin (50 nM) (Figure 1A).

Previous studies have shown that insulin and ATP stimulate distinct PI3-kinases in U937 cells (40, 27); insulin being coupled to the p85-dependent PI3-kinase whereas ATP activates the G-protein βγ subunit stimulated PI3-kinase. The response observed for FcγRI aggregation was, therefore compared in these same cells to that observed for insulin and ATP. The sustained rise in PIP_3 observed after FcγRI aggregation was not observed for either insulin or

ATP. Thus, insulin stimulated a rapid, transient increase of PIP_3 which quickly returned almost to basal levels within two minutes of receptor stimulation (Figure 1B). The time course for PIP_3 levels following ATP stimulation were similar to that for insulin although the reduction after the peak was less rapid than that observed for insulin but, unlike FcγRI aggregation, levels reached basal within two minutes.

Figure 5.1 PI3 kinase is activated by FcγRI aggregation in IFN-γ U937 cells.



The time course of activation differs from that observed for either tyrosine kinase or G protein stimulated PI3-kinase.

A. PIP_3 production in IFN-γ primed U937 cells following aggregation of FcγRI (XL). Concentrations were compared to cells in which no crosslinking antibody (No XL) was added and in cells treated with 50nM wortmannin (Wrt). The data shown are the mean ± the standard deveation of triplicate measurements for each time point and are derived from three separate experiments.

B. PIP_3 concentrations in IFN-γ primed U937 cells following stimulation of tyrosine kinase activated PI3-kinase with insulin (10 μg/ml) or G protein activated PI3-kinase with ATP (100μM). The data shown are the mean ± the standard deveation of triplicate measurements for each time point and are derived from three separate experiments.

5.4.2 FcγRI cross-linking stimulates tyrosine kinase-dependent PI3-kinase activities

As FcγRI has been shown to signal through the recruitment of non-receptor tyrosine kinases (41,42), the relationship of PI3-kinase to tyrosine kinase activation was investigated. The p85 subunit was rapidly tyrosine phosphorylated after FcγRI aggregation as phosphotyrosine could be detected within 30 secs of adding cross-linking antibody to the cells. This phosphorylation was attenuated in cells pretreated with 0.1mM genistein and was abolished in cells treated with 0.37mM genistein (Figure 2A).

The effect of inhibiting tyrosine kinases on PI3-kinase activation was measured. Pre-incubation of cells for 30 minutes with genistein (0.37 mM) completely blocked the increase of PIP_3 after receptor aggregation (Figure 2B). Consistent with the phosphotyrosine data, the lower concentration of genistein (0.1mM) did not abolish activity but reduced peak PI3 kinase activity by 68 ± 4 %. Three other tyrosine kinase inhibitors (2μM lavendustinA, 160 μM tyrophostin A23 or 28 μM tyrphostin A25) all inhibited peak PI3 kinase activity (Figure 2C).

This data indicates that all of the increase in PI3-kinase activity following FcγRI aggregation is downstream of the activation of tyrosine kinases.

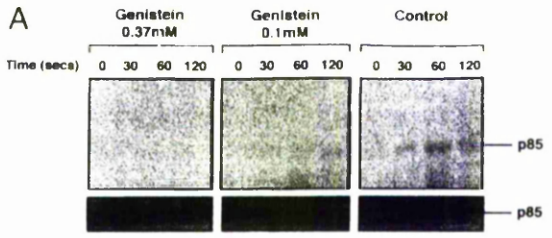
Legend for figure 5.2

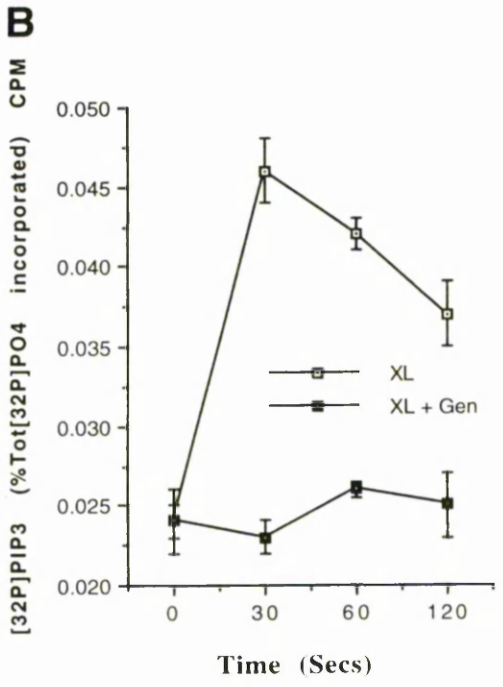
A. Western blot of immunoprecipitates of p85 probed with anti-phosphotyrosine antibody. Cells were harvested before aggregation of FcγRI and 30 sec, 1 min and 2 min after aggregation of the receptor. p85 was immunoprecipitated from cell lysates and the Western probed for presence of phosphotyrosine (upper panel). Untreated cells were compared to cells pretreated with either 0.1 mM genistein or 0.37 mM genistein. The blot was then stripped and probed with anti-p85 (lower panel).

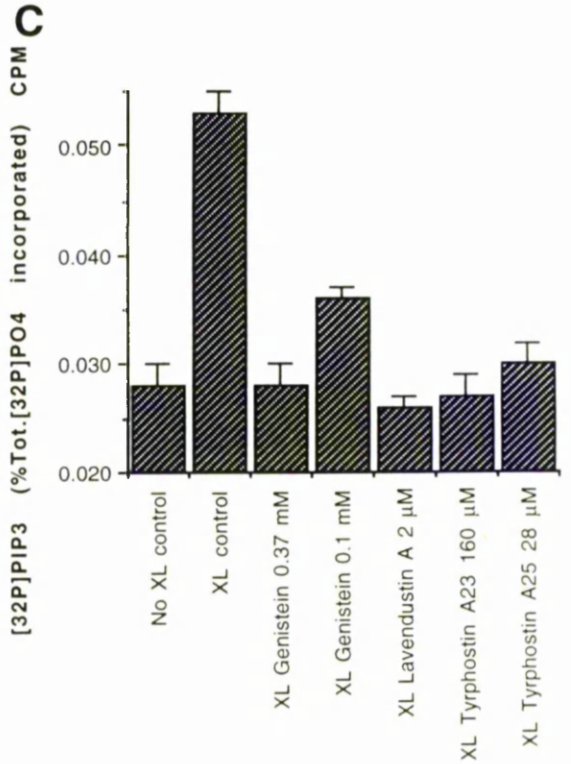
B PIP_3 production was measured in cells pretreated with genistein (0.37mM) for 30 mins prior to aggregation of FcγRI (XL + Gen) and compared to untreated cells (XL). The data shown are the mean $\pm$ the standard deviation of triplicate measurements for each time point and are derived from three separate experiments.

C Peak PIP3 production (30 secs) was measured in cells pretreated for 30 mins with various tyrosine kinase inhibitors and compared to control levels (XL control). Genistein was used at two concentrations - 0.37 mM and 0.1 mM; Lavendustin A - 2 μM, Tyrphostin A23 - 160 μM and Tyrphostin A25 - 28 μM. The data shown are the mean $\pm$ the standard deviation of triplicate measurements for each time point and are derived from five separate experiments.

Figure 5.2 Inhibition of tyrosine kinase activity blocks FcγRI-stimulated PI3 kinase activity.





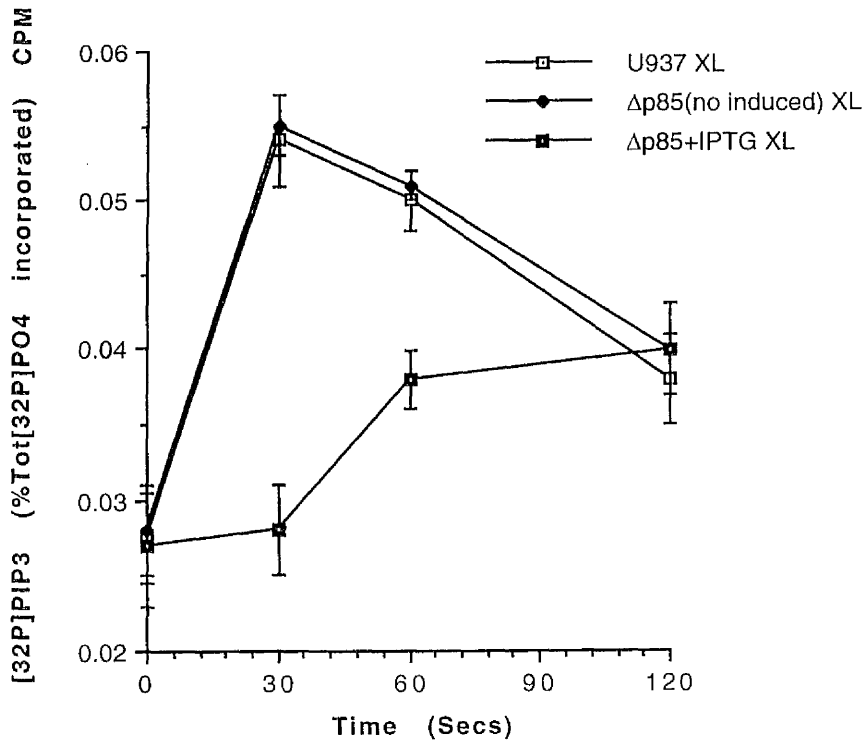


5.4.3 FcγRI cross-linking stimulates p85-dependent and -independent PI3-kinase activities

To investigate whether the PI3-kinase activity detected upon FcγRI stimulation was due to the activation of a p85 dependent PI3-kinase, the type usually associated with tyrosine kinase activation, a U937 cell line was used which had been stably transfected with an IPTG inducible dominant negative form of p85 (U937:Δp85). This dominant negative protein lacks the binding site for the p110 catalytic subunit of PI3-kinase and so when over-expressed will oblate p85 mediated PI3-kinase associations with other signalling components (40).

First, the kinetics of PIP_3 production was compared between the wild type U937 cells and the non-induced U937:Δp85 cells. This showed that in both cell types the temporal pattern of stimulation of PIP_3 levels in the cells was identical. Thus, peak levels were observed at 30 seconds but values in both cell types remained elevated over 2 minutes (Figure 3). However, following over-expression of Δp85, the pattern of PI3-kinase stimulation was significantly altered (Figure 3). In U937:Δp85 cells induced to express Δp85, the peak level of PIP_3 was delayed; significant stimulation was only detectable after one minute (Figure 3). However, two minutes after receptor aggregation similar levels of PIP_3 levels were measured for both Δp85-expressing and wild-type cells (Figure 3). Overall, the amount of PIP_3 generated after receptor aggregation was reduced and this reduced level of PI3-kinase was commensurate with the loss of the early, rapid peak of PI3-kinase activity observed in p85-functional cells.

Figure 5.3 Expression of a dominant negative form of p85, Δp85, abolishes only the early peak of PIP_3 observed after FcγRI aggregation.



The time course of FcγRI stimulated PIP_3 concentrations were measured in cells induced to express the dominant negative form of p85 (Δp85 + IPTG XL) and compared to the same transformed cells not induced to express (Δp85 (no induced) XL) and wild type U937 cells (U937 XL). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from three separate experiments.

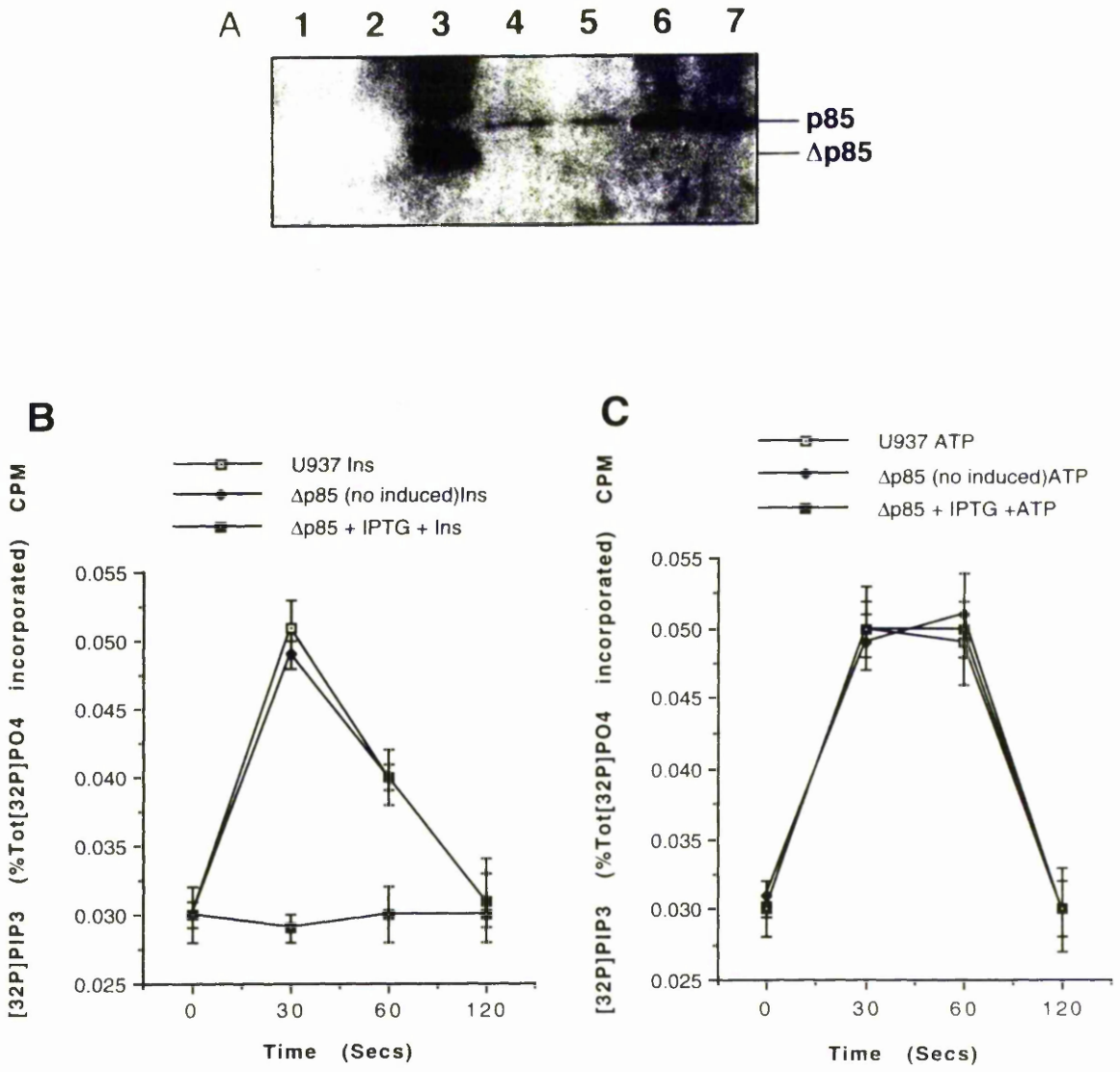
The correct functioning of the dominant negative Δp85 protein was shown in two ways. First, over-expression of the truncated Δp85 was verified by Western blot analysis which showed the truncated form of p85 only following induction with IPTG (Figure 4A). The functional dominant negative role for this truncated p85 was verified by assaying PI3-kinase activity following stimulation of the cells by insulin or ATP. In keeping with the known requirement of insulin for p85 to couple to PI3-kinase, induction of the dominant negative Δp85 in these cells completely abolished the expected rise in PIP_3 after insulin treatment (Figure 4B). In contrast, the response to ATP was entirely unaffected by the presence of the truncated p85 (Figure 4C). This finding is consistent with the fact that insulin exclusively stimulates p85 mediated PI3-kinase in response to tyrosine phosphorylation events whereas ATP exclusively stimulates p110γ, the G-protein βγ stimulated (p85-independent) PI3-kinase isoform. The data also clearly supports previous observations on the exclusive coupling of these two types of receptors to distinct PI3-kinase isoforms.

Taken together, these results suggest that FcγRI aggregation stimulates both a p85-dependent PI3-kinase and a p85-independent PI3-kinase. The temporal relationship of the response in the Δp85 cells suggests that the p85-dependent activity is responsible for the rapid and transient peak of PI3-kinase activity and the p85-independent activity is responsible for the delayed activity observed two minutes after cross-linking.

Legend for figure 5.4

A. Western blot analysis of cells induced to overexpress Δp85 compared to non-induced cells. Whole cell extracts were run in a 10 % SDS-polyacrylamide gel, proteins were then transferred to nitrocellulose and blotted with an anti-p85 polyclonal antibody as described in Materials and Methods. Lanes correspond to: 1. IFN-γ primed U937 cells; 2. IFN-γ primed U937:Δp85 cells non-induced for the expression of Δp85; 3. IFN-γ primed U937:Δp85 cells induced to over-express Δp85; - Lanes 4 and 5. Loading of twice amount of protein compared to lanes 1-3 (lane 4. IFN-γ primed U937 cells; 5. IFN-γ primed U937:Δp85 cells non-induced for the expression of Δp85); Lanes 6 and 7. Loading of four times amount of protein compared to lanes 1-3 (lane 6. IFN-g primed U937 cells; 7. IFN-γ primed U937:Δp85 cells non-induced for the expression of Δp85).
Bands corresponding to wild-type constitutive p85 and Δp85 are indicated.
B. PI3-kinase coupled to tyrosine kinase activation was inhibited in cells expressing the dominant negative, Δp85.
Production of PIP_3 in induced (Δp85 + IPTG + Ins), uninduced cells (Δp85 (no induced) Ins) and wild type cells (U937 Ins) following treatment with insulin (10 μg/ml). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from four separate experiments.
C. Activation of G protein coupled PI3-kinase was unaffected by the expression of the dominant negative p85.
Production of PIP_3 in induced (Δp85 + IPTG + ATP), uninduced cells (Δp85 (no induced) ATP) and wild type cells (U937 ATP) following treatment with ATP (10 μM). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from four separate experiments.

Figure 5.4 Verification that Δp85 is expressed and functionally active.



5.4.4 FcγRI cross-linking activates p110γ, a G-protein βγ subunit dependent PI3-kinase

Since it appeared that aggregation of FcγRI stimulated both p85-dependent and -independent activities, we next investigated whether the p85-independent activity could be due to the stimulation of the G-protein βγ subunit dependent PI3-kinase, p110γ. This enzyme has previously been purified and cloned from U937 cells (27, 28) and pig neutrophils (29, 30). To do this anti-sense oligonucleotides were designed complementary to the *N*-terminal encoding region of p110γ mRNA to knock out the expression of this enzyme. Two oligonucleotides were designed; a 30-mer which included a phosphothiorate group at each linkage, and a 24-mer which only had phosphothiorate links at either end. Both of these oligonucleotides significantly inhibited ATP stimulated PI3-kinase activity in U937 cells, an activity known to be attributed to p110γ (Figure 5A; 43, 27). In cells loaded with the short oligonucleotide, peak ATP stimulated PI3 kinase activity was reduced to 52 $\pm$ 6 % of control whereas, in cells loaded with the 30-mer, peak kinase activity was reduced to 25 $\pm$ 2 % of control. Consistent with this observation, the level of p110γ was reduced by equivalent amounts in cells pretreated with the antisense oligonucleotides (cells treated with short oligonucleotide 57 % of control; cells treated with 30-mer 28 % of control). In contrast, a control oligonucleotide of random DNA sequence had no effect on ATP stimulated PI3-kinase activity (Figure 5A). The longer of the two p110γ antisense oligonucleotides was found to be the slightly more effective of the two oligonucleotides, probably due to it being more resistant to nuclease attack. Neither oligonucleotide influenced the rise in PIP_3 observed after insulin stimulation (Figure 5B) findings consistent with the fact that the insulin receptor couples exclusively to the p85-dependent PI3-kinase.

Treatment of the IFN-γ primed U937cells with p110γ anti-sense oligonucleotides altered the pattern of PI3-kinase stimulated activity after FcγRI aggregation (Figure 5C). The initial rapid increase in PIP_3 levels previously observed 30 seconds after receptor aggregation remained unaltered. However, in marked contrast to control oligonucleotide treated and untreated cells, PIP_3 levels had returned to basal levels within one minute; kinetics reminiscent of the exclusive coupling of the insulin receptor to the p85 system. Indeed, these results essentially are the inverse of those obtained with the Δp85 expressing cells where the initial peak of activation was lost but stimulation remained elevated after one minute (Figure 3).

Taken together these results indicate that FcgRI cross-linking results in initial activation of a p85-dependent PI3-kinase which lasts less than one minute, and this is followed by the stimulation of a G-protein bg subunit dependent PI3-kinase (p110g). This latter activity was found to be insensitive to pertussis toxin (Figure 5D) and so was unlikely to have been activated by G-proteins of the G_i type.

Legend for figure 5.5

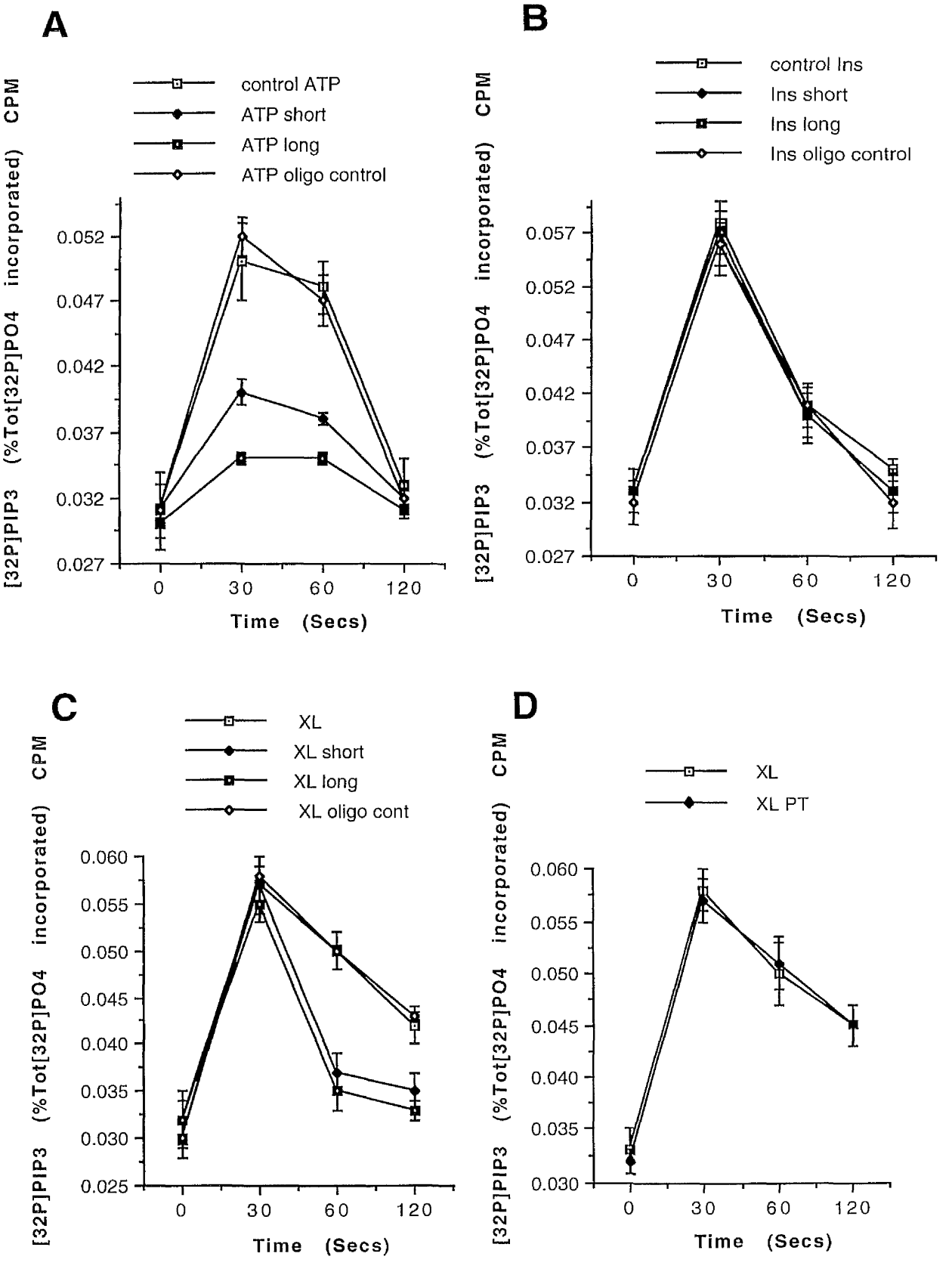
A. Treatment of cells with either antisense oligonucleotide to p110γ (ATP short and ATP long) significantly reduced PIP_3 following stimulation with ATP (100μM) (control ATP). A control jumbled oligonucleotide had no effect on ATP stimulated PIP_3 levels (ATP oligo control). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from three separate experiments.

B. Treatment of cells with either antisense oligonucleotide to p110γ (Ins short and Ins long) had no influence on the PIP_3 concentrations achieved after stimulaton with insulin (10 μg/ml). A control jumbled oligonucleotide had no effect on insulin stimulated PIP_3 levels (Ins oligo control). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from three separate experiments.

C. The initial peak in PIP_3 observed 30 secs following FcγRI aggregation was unaffected in cells treated with the p110γ antisense oligonucleotides. The delayed sustained rise in PIP_3 observed 2 mins after FcγRI aggregation (XL) was abolished by treating cells with this antisense oligonucleotide (XL short and XL long). The control jumbled oligonucleotide had no effect on any aspect of PIP_3 levels after FcγRI aggregation (XL oligo cont). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from three separate experiments.

D. Treatment of cells with pertussus toxin (XL PT) had no influence on PIP_3 concentrations observed after FcγRI aggregation (XL). The data shown are the mean ± the standard deviation of triplicate measurements for each time point and are derived from three separate experiments.

Figure 5.5 Treatment of cells with an antisense oligonucleotide to p110γ selectively abolishes ATP stimulated PI3-kinase and inhibits the delayed PI3-kinase activity after FcγRI aggregation.



5.5 DISCUSSION

We show for the first time, in this paper, that ligation of a single receptor can induce coupling to two distinct classes of PI-3Kinase: levels of PIP_3 rise very rapidly after aggregation of FcγRI in IFN-γ primed U937 cells due to the rapid and transient activation of a p85-dependent PI3-kinase. In contrast, the sustained elevation of PIP_3 observed following receptor aggregation appears to result from a delayed, longer lasting, but ultimately smaller p85-independent PI3-kinase activity. This latter activity was shown to be due to the activation of p110γ, a G-protein βγ subunit-sensitive form of PI3-kinase (29, 28). This is the first demonstration of a single receptor stimulating both tyrosine kinase and G-protein regulated PI3-kinases.

Activation of PI3-kinase appears necessary to mediate some of the effector functions for Fc receptors. Thus, inhibition of PI3-kinase with wortmannin inhibits antibody-dependent phagocytosis in U937 cells and COS cells (44, 45). It is interesting to note that although wortmannin inhibits Fc receptor mediated phagocytosis in U937 cells, it will not block endocytosis of immune complexes in these cells (data not shown). Previous studies have shown that PI3-kinase activity is increased in immunoprecipitates using anti-p85 and anti-phosphotyrosine antibodies in U937 cells following aggregation of FcγRI and FcγRII. This activation seemed to be secondary to tyrosine phosphorylation. Consistent with this, the data presented in this paper showed that the tyrosine kinase inhibitor, genistein, was able to block completely PI3-kinase activation (Figure 2). Similarly inhibition of tyrosine kinases with genistein has previously been shown to inhibit FcγRI-mediated phagocytosis in transiently transfected COS cells, but not FcγRI-mediated endocytosis in the same cells and in U937 cells (41, 46). Taken together, these results are consistent and suggest that PI3-kinase activation as a result of tyrosine phosphorylation events is

essential for FcγRI-mediated phagocytosis, but that endocytosis of immune complexes by FcγRI is independent of these signalling events.

The question relating to how FcγRI couples to both a p85-dependent and a G-protein subunit sensitive PI3-kinase remains to be answered. FcγRI contains no known signalling motif but has to recruit an accessory signalling molecule. In IFN-γ treated U937 cells, FcγRI recruits the γ chain which contains a cytoplasmic immunoreceptor tyrosine activation motif (ITAM) (8, 9). It therefore seems likely that the p85-dependent PI3-kinase activity is a result of a direct interaction between the SH2 domain of p85 and the ITAM of the γ chain, probably after tyrosine phosphorylation of the ITAM by a Src-type kinase or Syk (For review see 47). Indeed, the p85 regulatory subunit of PI3-kinase has been shown to bind with phosphopeptides of the γ chain ITAMs (48) but there is also evidence to suggest that p85 can directly interact with phosphorylated Syk via a YxxM motif on the Syk protein. It has been proposed for FcεRI signalling that Lyn becomes activated upon receptor clustering and tyrosine phophorylates the ITAMs of the γ chain, initiating the recruitment of Syk through its SH2 domain. Phosphorylated Syk can then activate other downstream signalling molecules including PI3-kinase (49). Recent studies have shown that Lyn is associated with both FcγRI and the γ chain independently of receptor cross-linking and that the level of the phosphorylation of this Src-type kinase increases upon receptor cross-linking (42). These studies also showed that Syk associates with the γ chain following FcγRI aggregation and the resultant phosphorylation of the g chain. The p85 regulatory subunit of PI3-kinase has been shown to bind with phosphopeptides of the γ chain ITAMs (48). Therefore it is probable that the p85-dependent PI3-kinase activity is recruited as a result of FcγRI recruiting the γ chain and subsequent tyrosine kinases, notably Lyn and Syk.

How FcγRI might recruit a heterotrimeric G-protein is less clear. The results presented here show that all PI3-kinase activity stimulated following FcγRI aggregation was abolished by pre-treating cells with genistein (Figure 2). This suggests that the activation of p110γ also occurs downstream of the activation of tyrosine kinases. It is possible that members of the Tec family of tyrosine kinases, for example Tec, Itk or Btk, could play a role in the activation of p110γ via a heterotrimeric G-protein since this family of enzymes all contain SH2 and PH domains. It is feasible that the SH2 domain of a Tec-type tyrosine kinase could associate with the γ chain (or a tyrosine phosphorylated protein such as Syk) and recruit a heterotrimeric G-protein whilst at the plasma membrane. This G-protein could then activate p110γ via its βγ subunits.

In conclusion we have shown in the present paper that two distinct PI3-kinase isoforms are activated following FcgRI aggregation in IFN-γ primed U937 cells; both p85-regulated and G-protein βγ subunit-sensitive PI3-kinase isoforms are activated. This is the first evidence to suggest that receptors such as FcγRI, which do not have an intrinsic signalling motif, can recruit both tyrosine kinase and G-protein-coupled intracellular signalling molecules to initiate distinct cellular responses. It has yet to be elucidated as to how FcγRI is able to recruit these different regulatory molecules and whether there are distinct physiological roles for the two PI3-kinases and their products.

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CHAPTER 6

GENERAL DISCUSSION

6.1 FcγRs ARE IMPORTANT SIGNAL GENERATING MOLECULES

FcγRs act as trigger molecules for inflammatory, cytolytic, endocytic and phagocytic activities of immune effector cells.

There are some common features in the signalling pathways initiated by Fc receptor aggregation. These common characteristics include the tyrosine phosphorylation of proteins (Scholl *et al* 1992, Liao *et al* 1992, Rankin *et al* 1993, Lin *et al* 1994), followed by rise in intracellular calcium levels (Rankin *et al* 1993). In the case of FcγRI, it has been shown that immune-complex aggregation of this receptor give rise tyrosine phosphorylation of proteins (including PLCγ1) (Liao *et al* 1992), and rise in intracellular calcium levels (Rankin *et al* 1993). However, the signalling events that occur between protein tyrosine phosphorylation and the rise in intracellular calcium is poorly understood. This lack of knowledge is due, at least partly, to the fact that the signalling pathways derived from monocytes and macrophages harvested under different condition are very heterogeneous in nature. In order to overcome this problem we have used the human monocytic cell line U937 (Harris & Ralph 1985). This cell line allows controlled differentiation with dbcAMP from monocyte phenotype to a more macrophage phenotype (Sheth *et al* 1988). Hence, they are widely used as a model to study, and better understand, the cellular events that may be happening when blood monocytes differentiate into tissue macrophages.

6.2 DIFFERENTIAL SIGNALLING PATHWAYS INITIATED BY Fcγ RECEPTORS

6.2.1 Novel pathway in IFN-γ primed cells

As PLCγ1 has been shown to be tyrosine phosphorylated following Fcγ receptor aggregation (Liao *et al* 1992), activation of PLC was measured in U937 cells following formation of surface immune complexes. In cells differentiated with dbcAMP to a more macrophage phenotype, a prompt and transient rise in $InsP_3$ was observed after immune complex aggregation of FcγR. However in cells primed with interferon-γ, no increase in $InsP_3$ could be detected. Since $InsP_3$ is transient by nature, it was possible that the $InsP_3$ peak had been missed as a result of sampling time. Lithium chloride was therefore used to assay total InsP accumulation over time. Lithium chloride prevents breakdown of InsP and can be a valuable tool in situations as here where there is concern that $InsP_3$ production is transient (Plevin *et al* 1991, Briscoe *et al* 1994). However, no accumulation of InsP could be detected in IFN-γ primed cells. This data suggests that PLC is not activated by immune complexes in these cells. This finding raises two issues. First, it is at variance with the reported tyrosine phosphorylation of PLCγ1. Second, it implies that the calcium transients observed in the cells arise through an $InsP_3$ independent route.

Tyrosine phosphorylation is widely accepted as a marker of activation. However, there are a number of examples where proteins are phosphorylated but no increase in activity can be observed. A classical example is PLCγ. It has been shown that phosphorylation of Tyr-783 in PLCγ is essential for stimulation of enzymatic activity in NHI-3T3 cells stimulated with PDGF (Kim *et al* 1991). Yet in many cases, the tyrosine phosphorylation of PLCγ

does not correlate with enzymatic activity; certain extracellular signals lead to weak tyrosine phosphorylation of PLCγ and strong production of InsP3, while other signals cause strong tyrosine phosphorylation of PLCγ but only very low levels of InsP3 are produced (Ree & Bae 1997). Thus, tyrosine phosphorylation *per se* cannot solely be used as a measure of enzymatic activation.

Cytosolic calcium concentration rise from two mechanisms; release of calcium from intracellular stores and entry of calcium from outside the cell. In IFN-γ primed cells, the rise in cytosolic calcium observed after formation of surface immune complexes, is transient in nature, typically lasting 1-2 minutes. Studies using manganese quench of Fura 2 or extracellular EGTA to chelate calcium in these IFN-γ primed cells have indicated that calcium entry contributes very little to the overall changes in cytosolic calcium concentrations. In immune cells, InsP3 is considered the major second messenger involved in release of calcium from stores (Liao *et al* 1992, Rankin *et al* 1993). InsP3 binds to the InsP3 receptor and initiates calcium release (Irvine 1990, 1991). However, the data presented in this thesis implies that in these cytokine primed U937 cells, calcium release from intracellular stores is mediated through an alternative mechanism. Work performed as part of this thesis has identified a novel pathway capable of calcium release through an InsP3 independent route.

In The absence of a measurable increase of InsP in IFN-γ primed U937 cells, an alternative activation pathway needed to be defined. Our first clue resulted from measurement of DAG. An equivalent increase in DAG was observed in both IFN-γ primed cells and in dbcAMP differentiated cells. At first sight, this was surprising as no PLC activation was detected in IFN-γ treated cells and

DAG is generally considered the product of PLCγ1 hydrolysis of PIP_2 (Cockcroft & Thomas 1992, Lee & Rhee 1995). However, DAG can also arise following the activation of PLD, which produces phosphatidic acid. This is subsequently hydrolysed by the action of phosphatidic acid phosphohydrolase to produce DAG (Exton 1994, Liscovitch & Cantley 1995, Hueng & Postle 1995). Although the stereoisoform of the two species of DAG differs (Pettitt *et al* 1997, Hodgkin *et al* 1998), the assay used cannot distinguish between them. The fact that the DAG arose as a result of PLD activation was confirmed by the use of butan-1-ol. As mentioned above, PLD generation of DAG is a two step reaction; first PLD breaks down phosphatidyl choline to generate phosphatidic acid and choline, then via the action of phosphatidyl-phosphohydrolase (PPH) phosphatidic acid is converted to DAG. In this reaction water acts a donor to the enzyme. However, primary short chain alcohols are much better donors than water, and, in the presence of such an alcohol, PLD will produce a phosphatidyl-alcohol, which is metabolised much more slowly than phosphatidic acid, and no DAG is produced (Hueng & Postle 1995, Pettitt *et al* 1997, Hodgkin *et al* 1998).

Phosphatidic acid is the immediate product of PLD activation and there is increasing evidence that this molecule may act as a second messenger. The most convincing evidence that phosphatidic acid has second messenger functions comes from the demonstration that PLD activation is an essential step in the stimulation of actin stress-fibre assembly, following activation of LPA receptors in PAE cells (Cross *et al* 1996). No other phospholipase is known to be activated in this situation. Furthermore, when butan-1-ol is present (causing generation of phosphatidyl-butanol rather than phosphatidic acid), LPA-stimulated phosphatidic acid accumulation and stress-fibre

formation are both inhibited. Exogenous phosphatidic acid induces stress-fibre formation even in alcohol-treated cells (Cross *et al* 1996).

The DAG product of PLD-PPH activity is a different stereoisoform to that of PLCγ1. There is considerably debate as to whether this DAG species is able to activate conventional isoforms of protein kinase C (Pettitt *et al* 1997, Hodgkin *et al* 1998). When measured by an assay that measures overall PKC activity, DAG derived from PLD did not seem to influence PKC activity in IIC9 fibroblasts. However, it was later suggested that this DAG is able to stimulate the atypical PKC_{ε} (Leach *et al* 1991, Ha & Exton 1993). However, this conclusion is questionable since both PLD and PLC could have been activated in these cells. In LPA-stimulated PAE cells, in which PLD is the only phospholipase known to be activated and only PLD-derived DAG is formed, LPA neither stimulated total PKC activity nor enhanced membrane translocation of any PKC isoform. At least in PAE cells, PLD-derived DAG does not seem to activate PKC (Pettitt *et al* 1997).

Sphingosine kinase (SK) was activated following formation of surface immune complexes in IFN-γ treated U937 cells. This sphingosine kinase activation was definitely demonstrated to be downstream of PLD activation. This finding correlates with in vitro studies where sphingosine kinase has been shown to be activated by phosphatidic acid (Olivera *et al* 1996). Sphingosine kinase yields sphingosine-1-phosphate as its product and assay of IFN-γ primed U937 cells detected this product after receptor aggregation. The role of sphingosine-1-phosphate (S-1-P) in causing calcium release from stores is controversial (Ghosh *et al* 1990, Mattie *et al* 1994, Wu *et al* 1995, Postma *et al* 1996). A number of reports have shown calcium transients when S-1-P is applied to cells. However, S-1-P receptors are found in the plasma membrane

(Wu *et al* 1995, Postma *et al* 1996, Lee *et al* 1998). These are classical G protein coupled receptors and are able to activate PLC thereby possibly releasing calcium through the $InsP_3$ receptor. However, a direct role for sphingosine products has been strengthened by the recent cloning of the SCaMPER receptor (Mao *et al* 1996). The work described in this thesis provides additional support for this novel mechanism of calcium release. Treatment of cells with butan-1-old to block the product of PLD activity, or DHS to inhibit sphingosine kinase resulted in diminished or oblated calcium transients in response to receptor activation.

Activation of PLD and SK was dependent on tyrosine kinase activation as genistein, an inhibitor of tyrosine kinases, blocked PLD and SK activation in a concentration dependent manner. There are three forms of PLD which differ in their activation mechanisms. Two of these have been cloned (Hammond *et al* 1997, Kotaki & Yamashita 1997, Colley *et al* 1997). It is unclear from the work presented here which form of PLD is activated in this pathway although work is continuing to investigate this. However, PI3-kinase activation was demonstrated in IFN-γ primed cells and this activation was tyrosine kinase dependent. Subsequent studies using wortmannin and LY290043 have shown that PI3-kinase lies upstream of PLD activation. Wortmannin is known to act on other enzymes at high concentrations (Bonser *et al* 1991, Cross *et al* 1995). Indeed, wortmannin (100mM) is capable of inhibiting PLD activity directly (Bonser *et al* 1991). However, the concentration of wortmannin (50nM) used to determinate the relationship of PI3-kinase activation to PLD activation was below this threshold and was demonstrated to have no direct effect on PLD activity following stimulation by phorbol esters.

Measurement of PI3-kinase activity in IFN-γ primed cells revealed a very unusual profile; activation was very prolonged, lasting for at least 15 minutes after immune complex aggregation of FcγRI. Two isoforms of the Class I PI3-kinase exist and have been defined in U937 cells (Hiles *et al* 1992, Hu *et al* 1993, Vanhaesebroeck *et al* 1997). One is activated by tyrosine kinases through the binding of the adaptor protein p85 to phosphorylated SH2 domains. The other is activated by βγ subunits of heterotrimeric G proteins (Stoyanov *et al* 1995, Stephens *et al* 1997). Insulin, which acts through tyrosine kinases, and ATP, which acts through a seven transmembrane domain receptor, are classically used to define these two enzymes. In this study, both the tyrosine kinase dependent form of PI3-kinase and the βγ dependent form of PI3-kinase were found to be activated by Fcγ receptor aggregation in IFN-γ primed U937 cells. The initial peak was p85 dependent whereas the delayed rise was dependent on the βγ activated form of the enzyme. The mechanisms underlying this dual activation remains to be resolved, but may well relate to the unusual second messengers generated in these cells. The findings reported here for IFN-γ primed cells contrast with preliminary studies in dbcAMP differentiated cells where PLCγ1 activation is solely dependent on the p85 (tyrosine kinase) dependent form of PI3-kinase. Direct measurement of PI3-kinase has shown that all the PI3-kinase activation can be explained as the p85, tyrosine kinase dependent form. In these cells, no βγ activation form of PI3-kinase could be detected.

6.3 DIFFERENTIATION DEPENDENT SWITCH IN SIGNALLING PATHWAYS BY FcγRI

The work presented in this thesis has shown that immune complexes activate distinct pathways depending on the differentiation status of U937 calls. Thus, in dbcAMP differentiated cells, immune complexes activate PLC whereas in IFN-γ primed cells a novel pathway is activated that induces the sequential activation of PI3-kinase, PLD and sphingosine kinase. These two pathways result in differing calcium transients. In dbcAMP differentiated cells, calcium oscillations are generated and cell population studies indicate that calcium store release is followed by stimulation of calcium entry (Davis, Sage & Allen) through the activation of ICRAC (Floto, Mahaut-Smith, Allen & Somasundram). In IFN-γ primed cells, a single spike in calcium is observed and store dumping is not associated with significant entry of calcium.

A differentiation dependent induction or suppression of the two pathways was unlikely as both pathways were demonstrated to be present in both cell types. Thus, PLC could be activated, using a different receptor, in IFN-γ primed cells and PLD activity could be measured, following stimulation with phorbol esters, in dbcAMP differentiated cells. An alternative explanation for the differentiation dependent switch in signalling pathways was therefore needed. The work presented in this thesis demonstrated that in IFN-γ primed cells, FcγRI recruits the γ chain for signal transduction whereas in dbcAMP differentiated cells, FcγRI recruits FcγRIIa for signal transduction. Furthermore, the γ chain appears to be coupled to the novel signal transduction pathway whereas FcγRIIa is coupled to PLC activation.

Tyrosine kinase activation is the initial event in both pathways. To activate tyrosine kinases; receptors either possess intrinsic kinase activity (e.g. NGFR, EGFR etc.) or a domain (in their cytosolic region) capable of recruiting cytosolic kinases. In cells of the immune system such domain is called **ITAM** (**I**mmune-receptor **T**yrosine **A**ctivatory **M**otif) (Reth 1989, Samuelson & Klausner 1992, Cambier 1995). The tyrosine residues of the ITAM are phosphorylated by Src-like kinases and these then act as high affinity sites for Syk kinase. Both the γ chain and FcγRIIa possess ITAMs although they differ substantially (Cambier 1995). In particular, the ITAM of FcγRIIa is unusually long as the intervening sequence between the tyrosine residues is 14 amino acids and not the typical 10 amino acids. It seems likely that the nature of the ITAM dictates the nature of the Src-like tyrosine kinase activated and that this in turn determine the signalling pathway initiated by immune complexes. The ability of FcγRI to switch signalling pathways reflects the fact that this receptor does not itself contain an ITAM in its cytoplasmic tail, but must recruit an accessory molecule for signal transduction. Thus, the ligand recognition domain and effector domain of the receptor complex are on separate polypeptide chains. This then allows the receptor to recruit alternate accessory molecules. Here, we have shown that this occurs for FcγRI and that the accessory molecule dictates the signalling cascade initiated by FcγRI. This has profound implications. First, it provides an explanation for the well-known heterogeneity of the response of monocytes and macrophages to immune complexes. Second, many receptors of the immune system display a similar organisation as the ligand recognition domain and effector domain lie on different polypeptide chains. It thus seems likely that other receptors may also employ a similar switch in signal transduction.

6.3.1 Consequences of the switch

The switch in signal activation pathway together with the associated change in calcium transients is likely to have profound effects on the nature of cell activation. In this thesis, the nature of the PKC isoforms activated by immune complexes was found to differ between the two cell types. Thus, in dbcAMP differentiated cells where calcium signals are prolonged, the calcium dependent PKC isoforms (α,β,γ) are activated. In IFN-γ primed cells, where the calcium response is transient, the atypical isoforms (δ,ε) and the novel (ζ) are activated. PKC activation is necessary to mediate phagocytosis (Zhelesnyak & Brown 1992, Zheng *et al* 1995, Karimi *et al* 1995). The results presented here provide an explanation for the finding that FcγRIIa mediated phagocytosis is calcium dependent whereas FcγRI mediated phagocytosis is calcium independent (Edberg *et al* 1995, Waite 1997).

Mechanisms of PKC activation in IFN-γ primed cells. If the stereoisoform of the DAG produced through PLD activation is inactive, then what is stimulating PKCs ?. Potential activators in this system could be phosphatidic acid, the primary product of PLD activity, which has been implicated in the activation of some of the PKC isoforms (Ohgushi *et al* 1997). Another potential candidate is PIP_3, the product of the Class I PI3-kinases, which has been shown to activate several isoforms of PKC (Toker *et al* 1993, Toker & Cantley 1997), the best example being the activation of PKC ζ (Nakanishi *et al* 1994, Standaert *et al* 1997).

Calcium transients are known to influence the transcription factors, NFAT and NF_kB (Crabtree & Clipstone 1994, Rao 1994, Baeurle & Henkel 1994, Dolmetsch *et al* 1997). Recent reports have shown that the frequency of

calcium oscillations influences transcription factor activation (Li *et al* 1998, Dolmetsch *et al* 1998). The switch in signal transduction pathways with the consequent switch in calcium transients therefore likely influences transcription factor recruitment and activation.

The work described in this thesis provides a possible mechanism to explain the differential activation of these two transcription factors. One signalling pathway (PLC) is linked to the generation of calcium oscillations whereas the other signalling pathway is linked to a single spike in calcium transients. The same receptor is capable of initiating the different calcium transients as it able to switch intracellular signalling pathways that dictate the calcium transients. The switch in signalling pathways is itself determined by the nature of the accessory molecule recruited by FcγRI.

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